Expression of cellular transporters of water and monosaccharides in the uterus and the placental transference zone in different gestational and non-gestational phases in the queen

Lluis Ferré Dolcet

Expression of cellular transporters of water and monosaccharides in the uterus and the placental transference zone in different gestational and non-gestational phases in the queen.

PhD Thesis
Dept. Medicina i cirurgia animal
Lluis Ferré Dolcet
Barcelona 2016

Director:
Mª Montserrat Rivera del Álamo

Director & Tutor:
Teresa Rigau i Mas

Universitat Autònoma de Barcelona

FACULTAT DE VETERINÀRIA

La Dra. Teresa Rigau i Mas, professora titular de la Unitat de Reproducció del Departament de Medicina i Cirugia Animals.

La Dra. Maria Montserrat Rivera del Alamo, investigadora post-doctoral de la Unitat de Reproducció del Departament de Medicina i Cirurgia Animals.

CERTIFIQUEN:

Que la tesi doctoral presentada per en Lluís Ferré Dolcet, sota el títol ***"Expression of cellular transporters of water and monosaccharides in the uterus and the placental transference zone in different gestational and non-gestational phases in the queen"*** per a optar el títol de Doctor, amb menció europea, per la Universitat Autònoma de Barcelona, s'ha dut a terme sota la nostra direcció. Així mateix, la considerem acabada i apta per a la seva defensa davant de la Comissió corresponent.

I perquè així consti a efectes oportuns, signem el present document.

Dra. Tesresa Rigau i Mas Dra. Maria Montserrat Rivera del Alamo

Bellaterra, Setembre de 2016.

Prof. Dr. Stefano Romagnoli, DVM, MS, PhD, Dipl. ECAR
Professor, Clinical Veterinary Reproduction
Department of Animal Medicine, Production and Health, University of Padova
Head, Small Animal Reproduction Service
Agripolis, Legnaro 35020 (Padova) Italy
☎ 39-049-827.2948 - fax 2954 - stefano.romagnoli@unipd.it

Legnaro (PD), July 18, 2016

To whom it may concern

Dr Lluis Ferré Dolcet has spent the period from January 5, 2016 to May 31, 2016 at the Veterinary Teaching Hospital, Department of Animal Medicine, Production and Health of the University of Padova, Italy. At that time Dr. Ferré Dolcet was a graduate student in animal reproduction at the University of Barcelona, Spain, and he came to our institution with the objective to be involved in some of the research projects that were ongoing at our department while still working on his own PhD thesis on the biology of aquaporins in queens reproductive tract. and, if possible, to get some further training in clinical reproduction of dogs and cats

Dr. Ferré Dolcet integrated immediately very well in our team of clinicians and got involved with a few of the research projects of our group, to which he contributed personally in a tangible way and for which we asked him to be a co-author of the related publications (n. 1 and 2). For some of these projects it was necessary to follow clinical cases, something which he did passionately and with a high degree of professionalism. Whenever it was necessary to interact with veterinary students Lluis Ferré Dolcet was always very gentle and kind, paying attention to student's needs and demonstrating a remarkable attitude in teaching.

Dr. Ferré Dolcet was determined to learn Italian in a professional way and insisted with all of us that we should correct him whenever he would make a mistake, and as a result he quickly became fluent in both spoken and written italian. Lluis Ferré Dolcet has a gentle and sensitive attitude toward human relationships, is always very respectful, presents himself well and never shows off his clinical and scientific background which is remarkable.

We all were very sorry to see him leave and hope to be able to collaborate with him again in the future.

Stefano Romagnoli

Head, Small Animal Reproduction Service
Veterinary Teaching Hospital
Department of Animal Medicine, Production and Health
University of Padova, Italy

1) Fertility and Reproductive Management of Norwegian Forest, Maine Coon, Persian and Bengal cats raised in Italy: a questionnaire-based study. Romagnoli S, Bensaia C, Ferré-Dolcet L, Sontas BH, Stelletta C. Submitted to the Journal of Feline Medicine and Surgery, June 2016
2) GnRH stimulation increases testicular blood flow in dogs. Kunik, B; Banzato, T; Milani, C; Ferré-Dolcet(*), L; Mollo, A; Romagnoli, S. Proc. International Symp Canine Feline Reproduction, Paris (France) June 22-25, 2016, page 96

Esta tesis se la dedico a mis mejores maestros:
A toda mi familia.

"I don't know
where I'm going
from here,
but I promise
it won't be
boring"

David Bowie.

Contents

Acknowledgements

"So I say
thank you for the music,
for giving it to me".
-ABBA-

Me dijeron una vez que no se debe agradecer a gente que sólo ha contribuido al tiempo y vivencias de esta tesis, sino que sólo se debe agradecer a aquellos que han trabajado en ella. Y yo, que siempre me he considerado una persona que hacía aquello que creía conveniente cuando creía conveniente, esta vez no quiero hacer caso a quien sentenció esas palabras; no por testarudez, sinó por reconocimiento.

Cierto es que esta tesis doctoral no hubiera sido posible sin las dos personas más importantes para ella. No puedo estar más agradecido por haber tenido no una, sino dos directoras de tesis para guiarme a través de este proceso con corage y entusiasmo.
Teresa, la teva dedicació a aquest camp es generosa indudablement per a tothom que hagi conegut i estic agraït per totes les oportunitats que m'has brindat durant aquests anys.
Montse, D&D. Agair-te de tot cor tot l'esforç i tot l'ensenyament continuu que m'has aportat, tots els bons i els difícils moments per els que hem passat durant tot aquest temps. Per tot això i moltes coses més que queden entre nosaltres, moltíssimes gràcies.

Agradecerle a Alejandro Peña. Le daría las grácias por ser un técnico excelente, pero no me quedaría tranquilo. Grácias por regalarme aquello que no recuperarás jamás, tiempo. Gracias por la paciencia de explicarlo todo mil veces, por corregir nuestros errores, por echarme una mano cuando la necesité y por ser mi fiel compañero de cafés. Gracias por las confidencias, las verdades y las risas; los paquetes de Oreo prohibidos, las quejas, las críticas y los viajes a fichar o a cargar agua destilada (cierto, en estos no te acompañaba tanto). Símplemente, gracias. Si los amigos son un tesoro, me llevo el oro.

A esos becarios; los de repro y los de otras disciplinas, gracias por la ayuda constante y los buenos [y malos] momentos. De entre ellos, debo destacar a algunos por ser merecedores de ello.

A Núria por ser mi primera compañera y amiga aquí, por enseñarme todo lo que sabía y por aprender a vitrificar (aunque yo empecé primero, hehehe). Por todas las risas, las lágrimas y los buenos momentos. No ens peguis més, que nosaltres t'estimem!

A Meritxell por incorporarse fantásticamente aún siendo la última en llegar. Esta tesis no hubiera sido posible sin su ayuda ni su valiosísimo tiempo (como becaria pringada que es y somos todos). Moltes gràcies per aconseguir els colors més macos allà on sigués; de tot cor.

A Henar, todas las risas y los cafés rápidos, las conversaciones sin sentido y todos los buenos momentos. ¿Qué sería la vida sin un microscópio en casa? En breves te copio.

A Martina Rocco, grazie per tutto. Sei un'amica che mi ha insegnato molto (anche un po' d'italiano). Il tempo con te e stato veramente una meraviglia. Adesso siamo un po'

più forte e la via un po' più piana. Aspetto vederti sempre; comunque ancora devo assaggiare la vera margherita e andare a fare un giro per la bella Napoli.
A Roser Morató, por enseñarme tanto a su lado y por dedicarme siempre un poquito de su tiempo a ayudarme en cualquier cosa. Por compartir mis inicios en este lugar de trabajo, por las risas, los gintonics, las estrellas michellin y por las situaciones en las que "casi nos cortamos las venas". Porque le debo mucho, y no sólo el compratir el amor por la buena cocina. Gràcies.

A Marc Yeste, por ser una cabeza numérica y entender todo aquello que no entendemos los demás. Por ser un genio y un currante donde los haya. Por todo su tiempo, esfuerzo y valía. Por confiar en este trabajo (a veces, incluso más que yo) y por ofrecernos tantas risas y buenos momentos. Moltes gràcies Marc.

A cada uno de los profesores de este servicio. A Teresa Mogas, Jordi Miró, Xus Palomo y Joan Enric Rodriguez, grácias por aportar vuestro grano de arena siempre que pudisteis. Los consejos, ideas, críticas y momentos. De todos se aprende algo, y estoy agradecido de haber podido aprender tanto a vuestro lado.

A todo el equipo de anatomía patológica de la Universitat Autònoma de Barcelona por echarme una mano con las complicadísimas inmunohistoquímicas; sobretodo a la Dra Rosa Rabanal, por su tiempo, sus consejos y por animarme a rpetirlas una y otra vez siempre con una sonrisa.

A tutto l'equipo di l'ospedalle didactico della università di Padova, professionali e anche studenti; tutti amici adesso. A Paolo Zuchini, technico di reproduzione, per tutto il suo tempo e dedicazione su tutto il lavoro fatto in laboratorio.
Ringraziare supretutto alla Dra Chara Millani per l'opportunità di lavorare con loro e per insegnarme tanto. E stato un piacere questo viagio con vuoi. Vi porterò sempre con me.

Voglio ringraziare veramente il Prof. Dr. Stefano Romagnoli, persona straodinaria dal lato umano e professionale che ha adesso la mia più profonda stima, rispetto e affetto. Nessun altro può dire di aver fatto per me così tanto senza pretendere nulla in cambio (soprattutto nel campo dell'insegnamento di questa lingua), o di avermi fatto sentire a mio agio e anche sicuro in tutto quello che ho fatto. Grazie per apprezzare cosí tanto la mia conoscenza e per aver messo tanta fiducia in me nel suo lavoro, dandomi la libertà di agire. Grazie per avermi fatto crescere in questa bellissima professione. Sarei lieto di lavorare insieme a lei in un prossimo futuro e di tenerci sempre in contatto. Perche il deslorelin e l'aquaporina, magari, si vedranno insieme in qualche studio. Grazie mille.

No puedo dejar en el olvido a todos aquellos que me ayudaron tantísimo tiempo atrás. Profesores de la carrera como Mariola Soler que se convirtió en una amiga o a Empar García Roselló, quien hizo que me enamorara de esto y me empujó a ello. También a todo el equipo del Máster en biología y biotecnología de la reproducción de la Universidad de Múrcia, tanto profesores como compañeros, por iniciarlo todo prendiendo la mecha y ser los mejores en ello.

También tengo suerte de tener un terrorífico grupo de amigos quienes me proporcionaron risas continuas y muy necesitadas diversiones. Porque no importa dónde

estéis, si en Valencia, París, Italia o aquí al lado. Siempre he podido contar con vosotros y os lo agradezco. He tardado mucho en terminar esta tesis, así que si os nombrara a todos, esto no acabaría nunca. Cada uno ya sabe el reconocimiento que merece.

Simplemente, no podría haber completado esta etapa sin mi familia; a ellos les debo las mejores lecciones. Ellos me enseñaron los básicos, que cierto es que sin ellos, no llegas a ninguna parte. Les agradezco el ayudarme en mi evolución diaria. Es cierto aquello que dicen que de una buena familia, saldrán personas buenas y honestas. Pero más cierto es, que de una buena familia saldrán personas felices. Porque no habría llegado hasta donde he llegado sin su fuerza, su confianza y el amor que siempre han depositado en mi. ¿Cómo no puedo estar agradecido a ellos? ¡Si son una familia ejemplar!
Tenemos problemas y discutimos [mucho] entre nosotros, como todas las familias, pero siempre hemos sabido sobrellevarlo. Cuando gané y perdí, cuando acerté y me equivoqué, siempre estuvieron por mí. Siempre se preocuparon por que fuera feliz y por que hiciera aquello que no me aburriera. Por mucho que lo neguemos, a fin de cuentas, una familia unida vence a cualquier cosa. Pensadlo bien; el diluvio universal fue un fracaso: quedó una familia con vida.

Durante todos estos años, mi primo Alberto Sainz me ha proporcionado risas continuas, buenos momentos y sabios consejos en el campo de la investigación (cada uno en su campo) y por ello, es merecedor de estar entre estas páginas.

A mi hermana, por todos los ánimos cuando necesité de ella y ahí estuvo.

A mi tía Mª Luisa por hacer ver que entendía algo de lo que le explicaba según avanzaba este proyecto y por animarme a no rendirme nunca; que lo difícil, da mejores y más sabrosos frutos. Por brindarme cientos de oportunidades, por su paciencia (Santa paciencia), los valores inculcados y por depositar su confianza en mí. Pero sobretodo, por actuar como un fortísimo pilar en la familia, ayudando en todo lo que puede, en lo que nadie le pide y sobretodo por hacerlo todo sin pedir nada a cambio. Muchísimas gracias.

A mi madre, por enseñarme siempre a darle el valor a las cosas, a darle a cada cosa la importancia que merece, por ayudarme siempre a salir de los momentos duros y a poder trasmitir el cariño siempre allá por donde vaya. Toda la vida ha estado ahí para darme una educación, una honestidad, un respeto, y aunque no lo parezca, una humildad; para que luego, agarre todo lo aprendido e intente sacarlo en mi trabajo y en todo en lo que sea partícipe. Gracias por entender mis ausencias, mis malos momentos y mis cabreos. Por los abrazos justo a tiempo, los ánimos y por saber que siempre necesité que estuvieras ahí para construirme como una persona fuerte y en quien soy en estos momentos (y no en el gili****** que podría llegar a ser). Se que ella está orgullosa de mí; y hoy quizás más que otros días. Por haberme dado la oportunidad de llegar hasta donde he llegado y así, poder transmitir todo lo que he hecho, gracias mamá.

Por último, agradecérselo a mi padre, que en paz descanse, por cuidarme y ayudarme a crecer siempre que estuvo; y quien ahora estaría más emocionado que nunca.

Y no puedo olvidarme de Luis Peixoto, a quien amo, quien me ha enseñado a aprender que yo no compito con nadie, que yo corro mi propia carrera y que gracias a eso, hoy en

día no tengo ningún deseo de jugar a ser mejor que nadie de ninguna manera, forma o estilo. Gracias por enseñarme solamente a mejorar, a ser mejor de lo que era antes. Porque tienes razón y no siempre debe ser un “yo, yo, yo y requete yo”.

Puedo decir que toda esta gente que quedará por siempre atrapada en estas páginas, me han enseñado a valorar el éxito; que para mi, quizás sea diferente a todos los demás. Para mí el éxito es la tranquilidad de saber que he vivido; quizás no todo lo que tengo que vivir, pero lo suficiente como para poder tomarme la vida con la serenidad de pensar que mañana quizás ya no esté aquí, en esta profesión o en esta vida. Esa filosofía medio suicida me mantiene despierto y feliz. Porque cada pasito que doy, por pequeño que sea, lo valoro tremendamente. Siempre he pensado que la única forma de ser feliz es ir actualizando los sueños, ya que cada nuevo nivel de tu vida, te exigirá una nueva versión de ti. Sólo hay que tenerle miedo a no haber vivido suficiente.

No considero que nadie sea más importante que nadie; igual que nada es más importante que nada, porque todo lo que he hecho en la vida, lo he hecho con amor. El mismo amor apasionado que siento por esta profesión y este trabajo.

List of papers

1. **Collaboration of aquaporin-1, -3 and -8 in queen reproductive tract may handle water transport on queen reproductive tract and placental transfer zone. Evaluation on different pregnancy stages associated to fetal development.**
2. **Aquaporin-2 expression in the uterus and placental transference zone at different gestational and non-gestational phases in the queen.**
3. **Glucose transporter 1 and 3 expression and location throughout different sexual and pregnancy stages in the queen reproductive tract: An immunoblotting and confocal microscope study.**

Abbreviations and symbols

Å ……..... Angstrom
AQP……...…… Aquaporin
Arg ……..…. Arginine
ATP ……. Adenosine triphosphate
CHIP……. Channel-like integral protein
Cys …….…….…. Cysteine
E2 ……..... Estradiol
ER …….. Estrogen receptor
GLUT…….……..…….….. Glucose transporter
GLY ……....……. Glicine
H^+ ……. Hydrogen ion
Hg^{2+}……..…….. Mercury
HIS …….. Histidine
HMIT ……..…….. H^+-myoinositol syntransporter
IGF-1 …….. Insulin growth factor –1
kDa ……..……. Kilodalton
L ……..……. Liters
LH ……....……... Luteinizing hormone
mRNA……. Messenger ribonucleic acid
NPA …….....……. Asparagine-proline-alanine
P4 …….……. Progesterone
RT-PCR ……...……. Reverse transcription polymerase chain reaction
SGLT ……...……. Sodium dependent glucose trensporter
Tyr …….…….……. Tyrosine

List of figures

Article 1

Article 2

Article 3

Todos los descubrimientos verdaderos
surgen del caos;
son resultado de dirigirse
hacia lo que parece
incorrecto
y ridículo
y tonto.

Chuck Palahniuk
- Invisible Monsters-

Abstract

The placenta is considered to be the major organ for the regulation of the exchange of solutes and nutrients between the conceptus and the dam. Because of this function, during pregnancy, both uterus and placenta concomitantly undergo several structural changes to prepare the endometrium for further implantation and fetal development. Fluid balance and glucose metabolism are essential mechanisms to prepare the epithelium and stroma for embryo implantation and are also involved in the fetal development by providing nutrition to the developing conceptus. These mechanisms are accomplished by water transporters, named aquaporins (AQPs), and glucose transporters (GLUTs). AQPs and GLUTs have been widely studied in the female reproductive tract of many species.

The aims of this study were:

- To determine the expression and location of AQPs 1, 2, 3 and 8 and GLUTs 1 and 3 in the queen uterus and placental transference zone.
- To determine a possible correlation between the expression of these proteins with serum levels of progesterone.
- To determine a possible correlation of these proteins between them at different sexual and gestational phases.
- To determine variations in AQP2 activity during queen pregnancy by evaluating the changes in the tyrosine phosphorylation levels of AQP2.

Queens were divided firstly into two groups: pregnant and non-pregnant queens. After that, pregnant queens were divided into 30, 40, 50 and 60 days of pregnancy according to the diameter of the fetal vesicle. Non-pregnant queens were divided into ovulated and non-ovulated queens according to serum levels of progesterone. Samples from endometrium and placental transference zone were evaluated by immunoblotting and immunohistochemistry techniques.

AQPs 1, 2, 3, 8 were present in the cells from both endometrial luminal and glandular epithelia and the placental transference zone during different sexual and gestational phases by western blotting and immunochemistry. AQPs 2, 3 and 8 have been located in the chorionic layers while AQP1 was absent, contrary to what has been described by other authors. No statistically significant changes in AQPs expression was observed at the different sexual and gestational phases. However, changes in the location of AQP2 and 8 related to serum progesterone levels were observed. Thus, when serum levels of

progesterone were low (<1ng/ml), these AQPs were located only in the cell membrane of luminal and glandular epithelia. When serum progesterone were high (>2ng/ml), these APQs were in both cell membrane and cytoplasm from luminal and glandular epithelia. Moreover, AQP2 activity was not regulated by tyrosine phosphorilation.

GLUT1 and 3 were also present in luminal and glandular epithelial cells from the endometrium and in the chorionic layer of the placental transference zone. No statistically significant changes in GLUTs expression were observed among the different sexual and gestational phases.

Finally, AQP2 showed a positive correlation with progesterone levels, while AQP1 and GLUT3 showed a negative correlation with serum progesterone levels. In addition, AQP1 and AQP3 showed a negative correlation between them in the evaluated phases.

In conclusion, the present study confirms the presence of AQPs 1, 2, 3, 8 and GLUTs 1, 3 in the queen endometrium and placental transference zone across different sexual and gestational phases as it has been previously described in other species, although with some specific differences.

La placenta está considerada como el órgano principal encargado del transporte de solutos y nutrientes entre madre y feto. Por su característica función, durante la gestación, tanto el útero como la placenta sufren diversos cambios estructurales para la preparación del endometrio para la futura implantación y desarrollo fetal.
El balance hidrológico y el metabolismo glucídico son mecanismos esenciales para la preparación del epitelio y del estroma para la implantación del embrión, estando también involucrados en el desarrollo fetal proporcionando una correcta nutrición al feto en desarrollo. Estos mecanismo son llevados a cabo mediante transportadores de agua denominados acuaporinas (AQPs) y transportadores de glucosa (GLUTs). Tanto las AQPs como los GLUTs han sido descritos ampliamente en el aparato reproductor femenino en una gran variedad de especies.
Los objetivos de este estudio fueron:

- Determinar la expresión y localización de las AQPs 1, 2, 3 y 8 además de los GLUTs 1 y 3 en el útero y la zona de transferencia placentaria de las gatas.
- Determinar una posible correlación de estas proteínas con los niveles séricos de progesterona.
- Determinar una posible correlación de estas proteínas entre ellas a diferentes fases sexuales y gestacionales.
- Determinar variaciones en la actividad de la AQP2 durante la gestación de la gata mediante la evaluación de cambios en su fosforilación de residuos de tirosina.

Las gatas se dividieron inicialmente en dos grupos: gestantes y no gestantes. Tras ello, las gatas gestantes se dividieron en 30, 40, 50 y 60 días de gestación según el diámetro de la vesicular embrionaria. Las gatas no gestantes se dividieron entre ovuladas y no ovuladas según sus niveles séricos de progesterona. Las biopsias realizadas de útero y zona de transferencia placentaria se evaluaron mediante técnicas de inmunoblotting e inmunohistoquímica.
Las técnicas de inmunoblotting e inmunohistoquímica revelaron la expresión de las AQPs 1, 2, 3, 8 en las células epiteliales del endotelio laminar y glandular además de en la zona de transferencia placentaria durante las diferentes fases sexuales y gestacionales. Las AQPs 2, 3 y 8 se expresaron en las capas coriónicas, mientras que la AQP1 no mostró ninguna expresión en las células del córion, contrariamente a lo anteriormente

descrito por otros autores. Estas AQPs no mostraron cambios estadísticamente significativos durante las diferentes fases sexuales y gestacionales. Sin embargo, se observaron cambios en la localización de las AQPs 2 y 8 relacionados con los niveles séricos de progesterona. Así, cuando los niveles de progesterona sérica eran bajos (<1ng/ml), éstas AQPs sólo mostraron su localización en la membrana citoplasmática de las células endoteliales del epitelio laminar y glandular. Cuando los niveles séricos de progesterona eran altos (>2ng/ml), éstas acuaporinas se encontraron tanto en la membrana citoplasmática como repartidas por todo el citoplasma del epitelio laminar y glandular. Además, la actividad de las AQP2 no estaba regulada por la fosforilación de tirosina.

Los GLUTs 1 y 3 también estaban presentes tanto en el epitelio laminar como en el epitelio glandular del endometrio y en las capas coriónicas de la zona de transferencia placentaria. No se encontraron cambios estadísticamente significativos en su expresión a lo largo de las distintas fases sexuales y gestacionales.

Finalmente, la AQP2 mostró una correlación positiva y significativa con los niveles séricos de progesterona, mientras que la AQP1 y el GLUT3 mostraron una correlación negativa con los niveles de progesterona. Además, las AQPs 1 y 3 mostraron una correlación negativa y significativa entre ellas en todas las fases evaluadas.

En conclusión, éste estudio confirma la presencia de las AQPs 1, 2, 3, 8 y de los GLUTs 1 y 3 en el endometrio y en la zona de transferencia placentaria de la gata a lo largo de diferentes fases sexuales y gestacionales como ha sido previamente descrito en otras especies a pesar de ciertas diferencias específicas.

La placenta està considerada com el principal òrgan encarregat del transport de soluts i nutrients entre la mare i el fetus. Per la seva funció característica, durant la gestació, tant l'úter com la placenta pateixen diversos canvis estructurals per a la preparació de l'endometri per la futura implantació y desenvolupament fetal.

El balanç hidrològic y el metabolisme glucídic són mecanismes essencials per a la preparació de l'epiteli i de l'estroma per a l'implantació del embrió, estant també involucrats al desenvolupament fetal proporcionant una adequada nutrició al fetus en creixement. Aquests mecanismes són duts a terme mitjançant transportadors d'aigua denominats aquaporines (AQPs) i transportadors de glucosa (GLUTs). Tant les AQPs com els GLUTs s'han descrit ampliament a l'aparell reproductor femení a una gran varietat d'espècies.

Els objectius d'aquest estudi van ser:

- Determinar l'expressió i localització de les AQPs 1, 2, 3 y 8 amés dels GLUTs 1 i 3 a l'úter i zona de transferència placentària de les gates.
- Determinar una possible correlació d'aquestes proteïnes amb els nivells sèrics de progesterona.
- Determinar una possible correlació d'aquestes proteïnes entre elles a diferents fases sexuals i gestacionals.
- Determinar variacions en l'activitat de l'AQP2 durant la gestació de la gata mitjançant l'evaluació de canvis a la seva fosforilació de residus de tirosina.

Les gates es van dividir inicialmente en dos grups: gestants i no gestants. Després d'això, les gates gestants es van dividir en 30, 40, 50 i 60 díes de gestación segons el diàmetre de la vesícula embrionaria. Les gates no gestants es van dividir entre ovulades i no ovulades segons els nivells sèrics de progesterona. Les biopsies realitzades d'úter i zona de transferència placentària es van evaluar mitjançant tècniques d'inmunoblotting i inmunohistoquímica.

Les tècniques d'inmunobloting i inmunohistoquímica van revelar l'expressió de les AQPs 1, 2, 3, i 8 a les cèl.lules epitelials de l'endoteli laminar i glandular amedés de a la zona de transferència placentaria durant les diferents fases sexuals i gestacionals. Les AQPs 2, 3 i 8 es van expresar a les capes coriòniques mentres que l'AQP1 no va mostrar cap tipus d'expresió a les cèl.lules del còrion, contrariament al que van descriure anteriorment diversos autors. Aquestes AQPs no van mostrar canvis

estadísticament significatius durant les diferents fases sexuals i gestacionals. D'altra banda, es van observar canvis de localització de les AQPs 2 i 8 relacionats amb els nivells sèrics de progesterona. D'aquesta manera, quan els nivells de progesterona sèrica eren baixos (<1ng/ml), aquestes aquaporines només estaven localitzades a la membrana citoplasmàtica de les cèl.lules endotelials de l'epiteli laminar i glandular. Quan els nivells de progesterona sèrica eren alts (>2ng/ml), aquestes aquaporines es van trobar localitzades tant a la membrana citoplasmàtica com repartides per tot el citoplasme de l'epiteli glandular i laminar. Ademés, l'activitat de l'AQPs no estava regulada per la fosforilació de tirosina.

Els GLUTs 1 i 3 també estaven presents tant a l'epiteli laminar com a l'epiteli glandular i a les capes coriòniques de la zona de transferència placentària. No es van trobar canvis estadísticamente significatius en la seva expresió al llarg de les diferents fases sexuals i gestacionals.

Finalment, l'AQP2 va mostrar una correlación positiva i significativa amb els nivells sèrics de progesterona, mentre que l'AQP1 i el GLUT3 van mostrar una correlació negativa. Ademés, les AQPs 1 i 3 van mostrar una correlació negativa i significativa entre elles a totes les fases evaluades.

En conclusió, aquest estudi confirma la presència de les AQPs 1, 2, 3, 8 i els GLUTs 1 i 3 a l'endometri i a la zona de transferència placentària de la gata al llarg de diferents fases sexuals i gestacionals com ha sigut previament descrit en altres espècies a malgrat de certes diferències específiques.

General introduction

Pregnancy is the physiological event during which one or more offspring develops inside a female. It is considered the result of a very complex process that includes the production and ovulation of healthy oocytes, the fertilization with semen of good quality, the implantation of the embryos in a healthy uterus and the maintenance of the pregnancy until parturition (Carson *et al.,* 2000; Jonhston *et al.,* 2001). Queen pregnancy lasts approximately 65 days from fertilization to parturition with different size litters (Jonhston *et al.,* 2001).

1.- PLACENTATION

For fetal development in mammals, placenta is mandatory. Placenta is considered an important communicating organ between the dam and the fetus that ensures the success of pregnancy by means of several changes as growth and differentiation (Carson *et al.*, 2000). Its principal function is to transport nutrients and waste products between the dam and the fetus (Damiano *et al.*, 2011). Placenta is in charge of the hemotrophic nutrition, meaning the transfer of nutrients from the dam to fetal blood, which is complemented with the histotrophic nutrition (maternal blood cells, uterine secretion uptake or cellular debris) (Enders *et al.,* 2006).

The placenta regulates the exchange between the fetus and the dam by simple diffusion (gas and water), facilitated diffusion (glucose, amino acids), active transport (sodium, potassium and calcium pumps), pinocytosis and phagocytosis (Enders *et al.*, 2006). Lipids are unable to cross the placenta. However, placenta is able to synthesize new lipid components for fetal use from hydrolyzed maternal triglycerides and phospholipids. Fat-soluble vitamins neither cross the placenta easily, while water-soluble vitamins can pass through without any difficulty (Senger, 2003). In addition to nutrients transport, the placenta has also an endocrine function and it is able to produce a variety of hormones, which are important for the maintenance of pregnancy and parturition induction (Senger, 2003).

Placenta is formed by a maternal component and a fetal component. The maternal component implies the modifications that the endometrium undergoes after implantation. As fetal components we can describe the amnion, allantoides and chorion.

The chorion, the fetal contribution to the placenta, is composed of several chorionic villi, which are projections of the chorionic surface that invade the maternal endometrium and are considered as the functional part of the fetal placenta.

The placenta can be classified, on the one hand, according to the number of layers that separate maternal and fetal blood. Thus, placentas can be classified as epitheliochorial, endotheliocorial and hemochorial (Senger, 2003).

a) Epitheliochorial: Characteristic of sow and mare (Fig.1). This kind of placenta is the least intimate. The maternal endometrial epithelium is intact as the chorionic villi epithelium is. Ruminants also have an epitheliochorial placenta, but in this case, an erosion of the endometrial epithelium exists that later grows again exposing the maternal capillaries to the chorion. It is termed as syndesmochorial placenta.

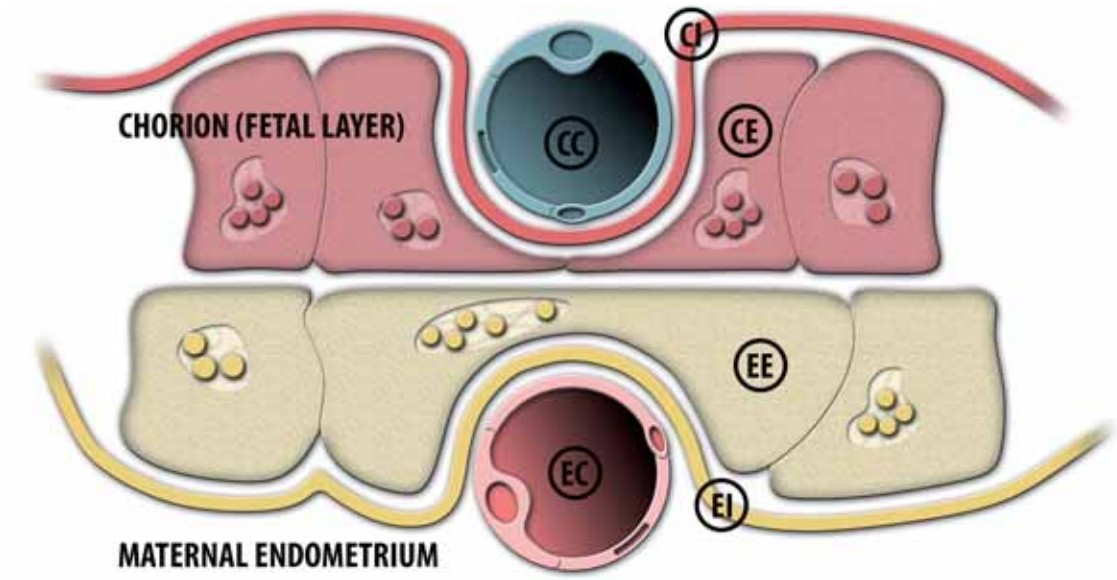


Fig.1 Epitheliochorial placenta. CC, Chorionic capillaries; CI, Chorionic interstitium; CE, Chorionic epithelium; EE, Endometrial epithelium; EI, Endometrial interstitium; EC, Endometrial capillaries. Image based on Senger. 2003.

b) Endotheliochorial: Characteristic of carnivores (Fig. 2). The endometrial epithelium is completely eroded and underlined by the interstitium. Because of that, the

chorionic epithelium has a direct exposition to the maternal capillaries. As pregnancy progresses, the endometrial epithelium disappears.

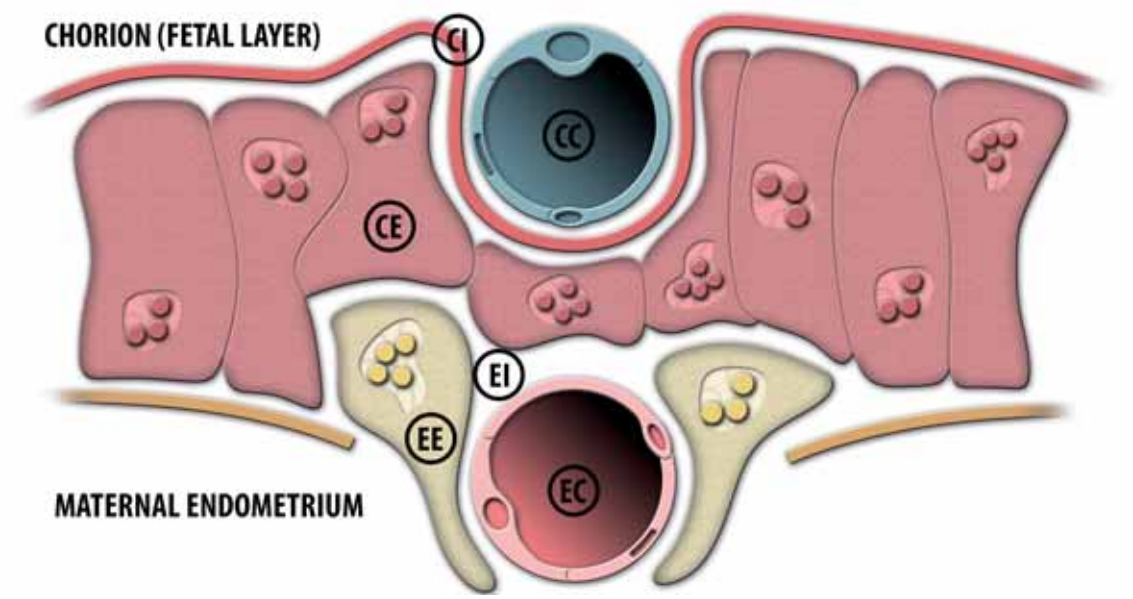


Fig.2 Endotheliochorial placenta. CC, Chorionic capillaries; CI, Chorionic interstitium; CE, Chorionic epithelium; EE, Endometrial epithelium; EI, Endometrial interstitium EC, Endometrial capillaries. Image based on Senger *(2003).*

c) Hemochorial. Characteristic of primates and rodents (Fig. 3). The chorion is in direct contact with the maternal blood. Nutrients and gases exchange occurs directly from maternal blood.

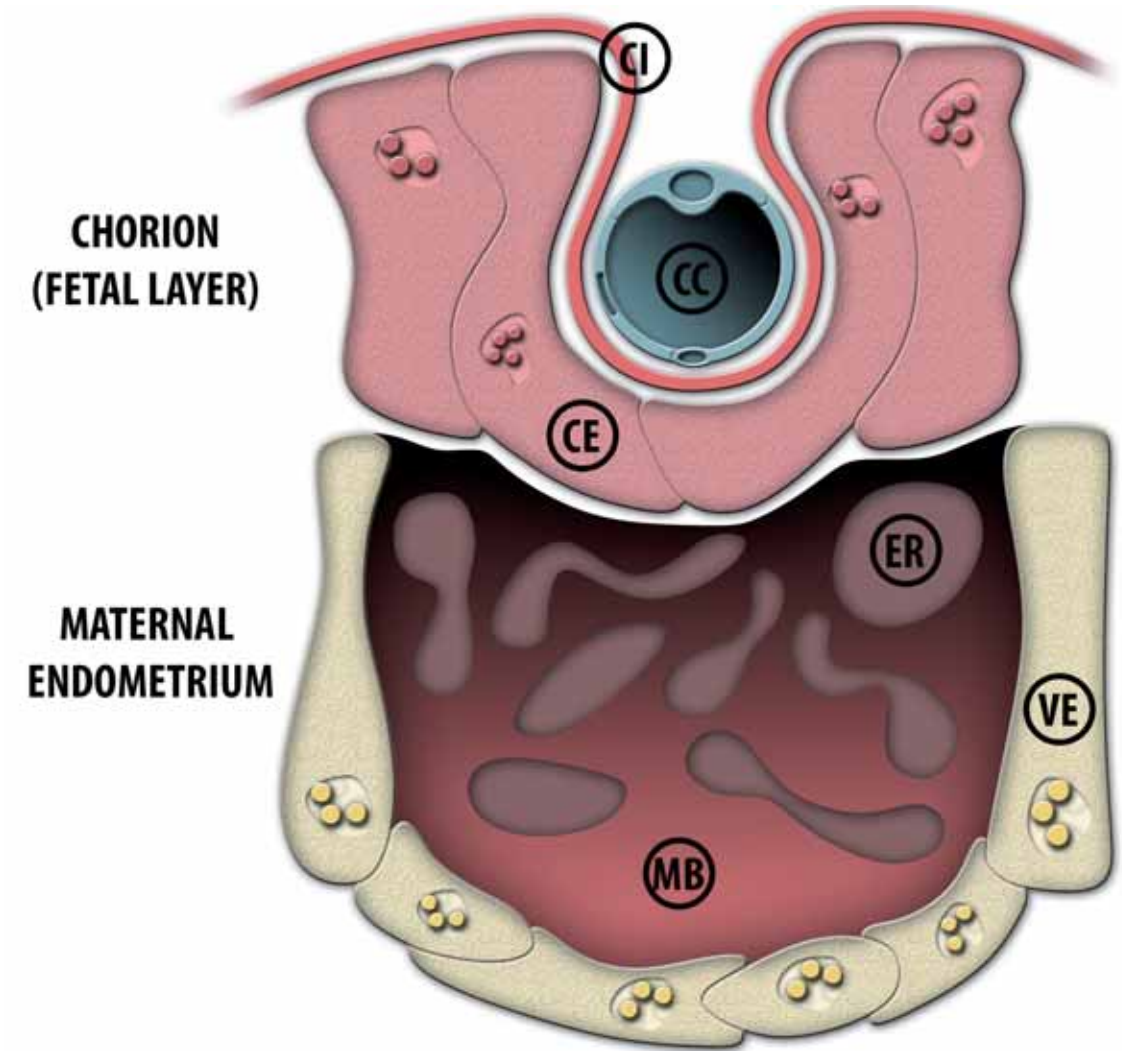


Fig.3 Hemochorial placenta. CC, Chorionic capillaries; CI, Chorionic interstitium; CE, Chorionic epithelium; ER, Erytrocytes; MB, Maternal blood; VE, Vascular endothelia. Image based on Senger *(2003).*

On the other hand, placentas can be also classified according to the chorionic villi distribution on their surfaces, providing a distinct anatomical appearance. Thus, placentas are classified in diffuse, zonary, discoid and cotyledonary (Senger, 2003).

1) Diffuse placentas are characteristic of sow and mare. Chorionic villi are evenly distributed all over the entire chorionic surface.
2) Cotyledonary placentas are characteristic of ruminants. The cotyledons are unions of trophoblast with the connecting tissue. Fetal and maternal cotyledons union forms the placentome, creating the caruncular regions of the uterus. Previous to the formation of placentomes, ruminant placenta is mostly diffuse,

until the chorionic villi starts to create crypts in the caruncular site originating the placentomes and transforming the placenta into a cotyledonary type.

3) Zonary placentas are characteristic of carnivores. Chorionic villi are distributed forming a girdle localized in the central area of the placenta. Laterally to this exchange area, pigmented bilateral ring-like regions composed by hematomas are present.
4) The discoid placenta is found in rodents and primates. The chorionic villi are distributed in one or two discs. The chorionic villi interface with the endometrium forming the area of exchange of waste and where nutrition takes place.

1a.- Carnivorous placentation

In carnivores such as dog and cat, as it has been stated above, placentation is defined as endotheliochorial zonary with a complete girdle in dogs and an incomplete one in cats (Miglino *et al.*, 2006) (Fig. 4).

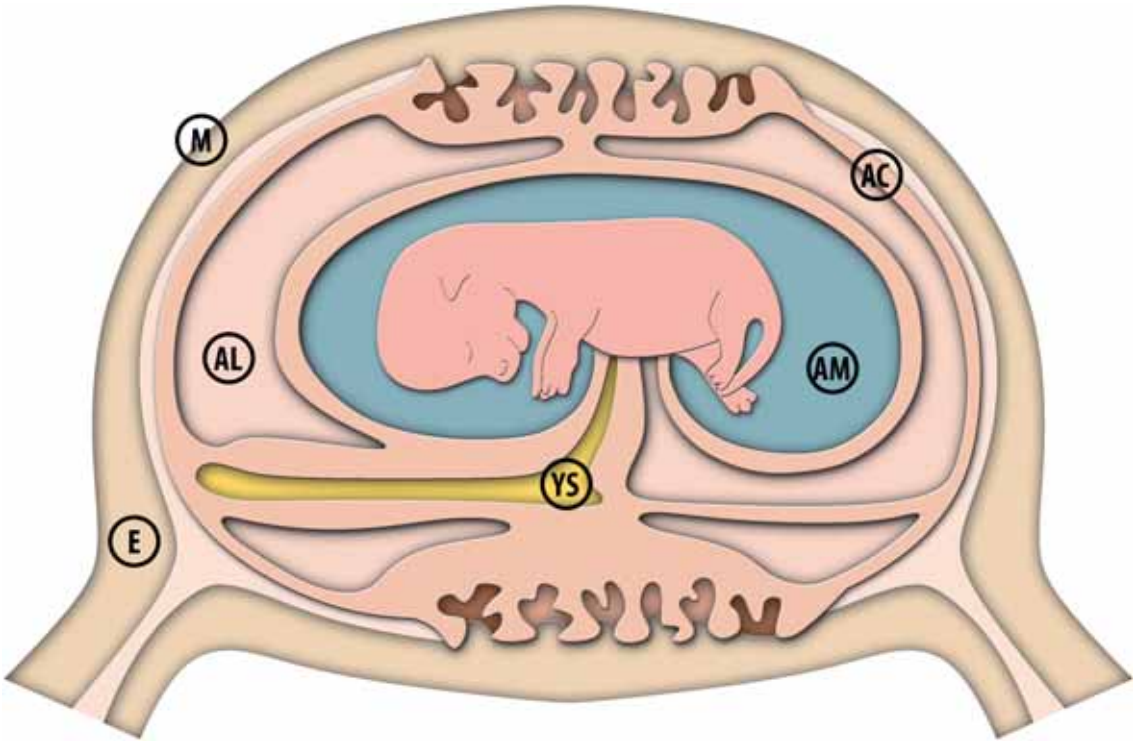


Fig.4 Carnivore placenta. M, Myometrium; E, Endometrium; AC, Allantochorion; AL, Allantois; YS, Yolk sac; AM, Amniotic cavity. Image based on Senger *(2003)*.

Zonary placenta includes a region of exchange, which forms a broad zone around the chorion and near the middle of the conceptus, named the placental transference zone. A second region, named paraplacenta, is bilaterally located at the ends of the transference zone. It consists in a highly pigmented ring composed of small hematomas and it is important for iron transport from the dam to the fetus (Senger, 2003). The allantochorion is considered a third placental zone, which is characterized by a poor vascularization and is involved in the direct absorption of materials of the uterine lumen (Senger, 2003) (Fig. 5).

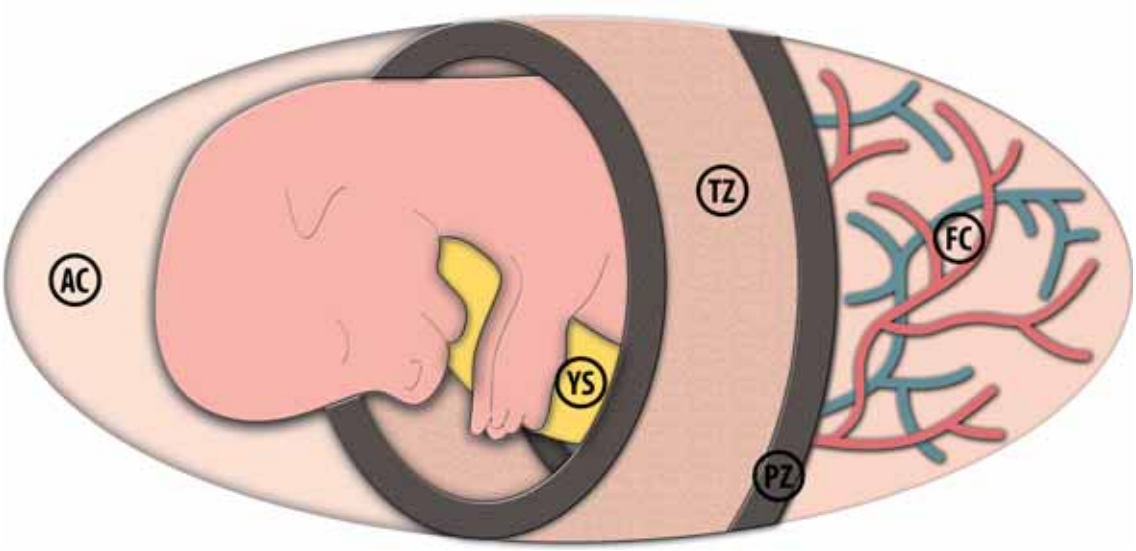


Fig.5 Carnivore placenta. AC, Allantochorion; YS, Yolk sac; PZ, Pigmented zone; TZ, Transference zone; FC, Fetal circulation. Image based on Senger *(2003).*

When the carnivore placenta is evaluated under the microscope, it can be defined as an endotheliochorial placenta, characterized by a complete erosion of the endometrial epithelium and the underlying interstitium. In this case, maternal capillaries are directly exposed to the chorionic epithelium. This type of placenta is so invasive that the endometrial epithelium around the maternal capillaries finally disappears.

In the domestic queen, once ovulation has ocurred, endometrial glands and myometrium modifications facilitate the implantation of the conceptus, which takes part 12-14 days after ovulation by means of the initial invasion of the trophoblasts (Leiser, 1979). After this event, most of the changes occur mainly in the endometrium. Before day 20th of

pregnancy, where the embryonic allantochorion membrane will provide the surface for attachments to the endometrium, the cat embryo nutrition is supplied by a choriovitelline placenta (Edwards *et al.,* 1993), creating the yolk sac and playing a significant role early in pregnancy. It is from mid pregnancy on when endometrial glands supplies histiotrophic nutrition. As pregnancy progresses to terminus, the chorionic placental labyrinth increases its thickness to ensure fetal nutrition (Aralla *et al.,* 2012).

2.- WATER TRANSPORT

Water is considered to be crucial in all processes for living cells and its movement across the plasma membrane is fundamental for water homeostasis during fetal development (Sha *et al.,* 2011). On the early '90s, a 28kDa protein was found in the red blood cells and renal tubules, and named CHIP 28 (Channel-like Integral Protein of 28 kDa) (Agre *et al.,* 1987). It was organized in tetramers and was selectively permeable to water, explaining the high permeability of the membranes where that protein was present (Agre *et al.,* 1987; Smith & Agre 1991; Preston *et al.,* 1992). This protein was finally named as aquaporin 1 (AQP1) after being cloned and sequenced by Peter Agre, who was awarded in 2003 with the Nobel Prize in chemistry for his work on aquaporins (Preston & Agre, 1992; Moon *et al.,* 1993; Agre *et al.,* 1993). Since this discovery, a lot of research has been performed and other proteins with the same characteristics have been discovered and named as aquaporins with a sequential number according to the order of discover. To date, a total of 13 aquaporins have been described in mammals.

2a.- Molecular structure, function and location of aquaporins (AQPs)

AQPs are regulated by many intracellular factors like pH and phosphorylation, mainly mediated by protein kinase A (Denker *et al.,* 1998). The structure of AQPs consists in six domains which are connected by five loops where each polypeptide (formed by a single chain with approximately 270 amino acids) has a terminal amino and carboxyl groups and are always located in the cell cytoplasm (Denker *et al.,* 1998). These loops can be extracellular loops (A, C, and E) and intracellular loops (B and D) (Yasui *et al.,* 1999). All AQPs are conformed by two very similar halves which are joined by the loop

C, which have a structural function (Saparov *et al.,* 2007), while B and E loops are essential for water permeability and pore formation (Tsukaguchi *et al.,* 1998) (Fig. 6). AQPs are tetrameric structures (Tsukaguchi *et al.,* 1998), but some of them can form smaller oligomers such as AQP4.

The function of most of the AQPs can be inhibited by mercury (Hg^{2+}) because of a cysteine residue (Cys-189) in the E loop. However, AQP 4 and 6 are activated by Hg^{2+} (Yasui *et al.,* 1999).

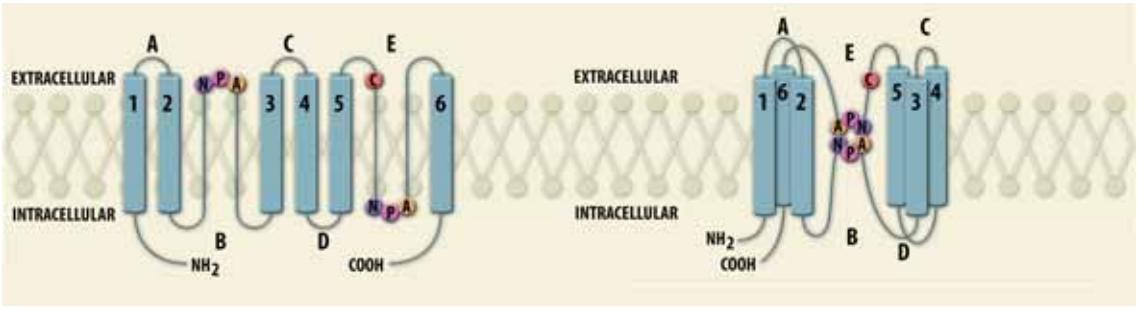


Fig.6 Molecular structure of aquaporins. The hourglass model (Jung *et al.,* 1994).

Aquaporins (AQPs) are considered small (about 28-35kDa) hydrophobic integral cellular membrane proteins with the functional role of water transport and permeability increase of the lipid bilayer membrane (Agre, 2004). Its water permeability is in the order of $3x10^9$ water molecules per second for AQP1 and closer digits to the other aquaporins (Ishibashi, 2009).

Aquaporins are highly selective to water transport preventing also the protons transport (Gomes *et al.,* 2009). The Arg-195 residue in the pore prevents the pass of protonated water (Ishibashi *et al.,* 1997) and a second barrier composed by the intracellular loops with the asparagine-proline-alanine (NPA) sequence reorients the water molecules to pass, disrupting interactions between molecules and preventing them to pass through (Shanahan *et al.,* 1999). In general, these water channels also prevent the pass of other anions because of its 2.8 Å measure (less than any hydrated ion), but in the aquaglyceroporins, an alternative GLY on HIS-180 is associated with a bigger pore with the capacity to transport glycerol and other solutes (Nielsen *et al.,* 1997b).

Based on its permeability, aquaporins have been classified in 3 different categories: (1) classical AQPs, (2) aquaglycerolporins and (3) superaquaporins.

Classical aquaporins include AQP0, 1, 2, 4, 5, 6 and 8. They are considered only water selective channels. Nevertheless, AQP6 is considered to be able to transport ions (Yasui *et al.,* 1999) and AQP8 is also permeable to ammonia (Saparov *et al.,* 2007).
Aquaglycerolporins include AQP3, 7, 9, and 10. In addition to be permeable to water, these AQPs are also permeable to urea and glycerol. Besides, AQP9 has the facility to transport also monocarboxylates, purines and pirimidines (Tsukaguchi *et al.*, 1998).
Finally, superaquaporins include AQP11 and 12. These AQPs are located in the cytoplasm and its permeability has not been fully determined yet (Ishibashi, 2009).

AQPs are very ubiquitous in mammals and usually are not restricted to a unique tissue. However, their function will be determined by their specific location in the cell types or organs (Gomes *et al.*, 2009). It has been shown that several organs such as brain, lungs, muscles, eye, ear, skin, adipose tissue, testis, uterus and placenta contain more than one AQP (Li *et al.,* 1994; Umenishi *et al.,* 1996; Ishibashi *et al.,* 1997; Nielsen *et al.,* 1997; Nielsen *et al.,* 1997b; Shanahan *et al.,* 1999; Johnston *et al.,* 2000).

First studies in AQPs were performed in kidney (Agre *et al.,* 2002). Kidney is an organ that contains approximately one million of nephrons that filter around 180L of plasma every day, in addition to water and other solutes reabsorption (Newman, 2008). From then on, AQPs have been found in a large number of other tissues and organs such as lung, pancreas, brain, gastrointestinal tract, eye, ear, immune system, skin, adipose tissue, muscles, uterus and testis (Li *et al.,* 1997; Ishibashi *et al.,* 1997b; Ishibashi *et al.,* 1997c; Frigeri *et al.,* 1998; Page *et al.,* 1998; Beitz *et al.,* 1999; Shananan *et al.,* 1999). Many clinical studies have shown that the failure on the function of AQPs turns into pathologies (Deen & Knoers, 1998; King *et al.,* 2000; Nielsen *et al.,* 2002) leaning on the importance of water trafficking for all biological processes. Moreover, it has been demonstrated, that some aquaporins are regulated by sex steroid hormones (Aralla *et al.,* 2009; Skowronski *et al.,* 2012; Klein *et al.,* 2013).

2b.- Aquaporins and the reproductive tract

Several AQPs have been widely described in both male and female reproductive tract of different mammal species such as human (Edwards *et al.,* 1993; Zhu *et al.,* 2009), rat (Braés *et al.,* 2005), mice (Anderson *et al.,* 2006), pig (Skowronski, 2010), horse (Klein *et al.,* 2013), sheep (Liu *et al.,* 2008), dog (Aralla *et al.,* 2009; Aralla *et al.,* 2012) and cat (Arrighi & Aralla, 2014).

2ba.- AQPs in the male reproductive tract

Several studies have demonstrated the presence of AQPs in the male reproductive tract. AQPs0, 1, 7, 8 and 9 have been recognized in the testis; AQPs 1, 3, 9 and 10 in the epididymis; AQPs 1, 9 and 10 in the efferent ducts; AQPs 1, 2 and 9 in the vas deferens and AQP1 and 9 in accessory glands of adult mammals.

Two AQPs have been found to be expressed in the rat testis (AQP7 and 8) suggesting that they may play a role in spermatogenesis (Ishibashi *et al.,* 1997b). Russel *et al.,* (1989) suggested that both AQP7 and 8 may contribute in the creation of seminiferous fluid. AQP7 expression intensity and location change depending on the stage of spermatogenesis (Suzuki-Toyota *et al.,* 1999; Calamita *et al.,* 2001a). In contradistinction to AQP7, AQP8 shows a consistent intracellular expression as well as all over the plasma membrane in every rat seminiferous tubule (Calamita *et al.,* 2001b). However, AQP8 is absent in human testis (Koyama *et al.,* 1998). Sertoly cells have been shown to express AQP0 (Hermo *et al.,* 2004) and AQP8 (Tani *et al.,* 2001). Leydig cells express AQP9 (Tsukaguchi *et al.,* 1998) and AQP0 (Hermo *et al.,* 2004) in rat.

AQPs have been also described in sperm cells of several species such as dog (Fatin *et al.,* 2008), boar (Prieto-Martinez *et al.,* 2014; Prieto-Martinez *et al.,* 2015), bull (Prieto-Martinez *et al.,* 2016) and cat (Arrighi *et al.,* 2010). Thus, AQP9 has been detected in rat spermatocytes (Tsukaguchi *et al.,* 1998), In addition, AQP7 is also expressed in rat epididymal sperm (Calamita *et al.,* 2001a). As AQP7 is considered an aquaglycerolporin, the role of AQP7 in sperm cryopreservation justifies further research (Huang *et al.,* 2006).

As it has been previously mentioned, AQPs are involved in the secretion of fluids at different levels. In the male reproductive tract, rat prostate and seminal vesicles express AQP1 (Brown *et al., 1993)*. In addition, rat prostate and Cowper gland express AQP9 (Pastor Soler *et al.,* 2001). Both aquaporins have a role in both secretory and absorptive functions of these organs (Brown *et al.,* 1993; Pastor Soler *et al.,* 2001).

In addition to secretion production, AQPs contribute also in the reabsorption of fluids. It has been described that seminiferous tubules secrete luminal fluid forward reabsorbed by efferent ducts, and water channels (AQP1, 9 and 10) may have a role in this trans-epithelial water movement (Clulow *et al.,* 1998).

Fisher *et al.* (1998) described the presence of AQP1 in basolateral membranes and apical endosomes of non-ciliated cells of the efferent ducts of rats and monkeys from late fetal stages until adulthood. Further, Zhou *et al.* (2001) suggested that other AQPs might be the responsible of water movement in the efferent ducts due to the fact that no alterations on water fluid movement were observed in AQP1-null mouse.

Moreover, AQP9 was detected to be present in the microvilli of non-cilliated cells (Pastor-Soler *et al.,* 2001; Oliveira *et al.,* 2005). In addition, Hermo *et al.* (2004) described the presence of AQP10 in the epithelia of efferent ducts and, along with AQP1, in the endothelial cells of vascular regions (Brandan and Hermo, 2002; Hermo *et al.,* 2004).

As it has been previously mentioned, steroid hormones regulate the expression of some AQPs. Ruiz *et al.* (2006) described that AQP1 and 9 expression is regulated by estrogen. In deficient estrogen receptor (ER) α-mice, the expression of these specific AQPs is decreased.

It has been also described that in the epididymis a huge fluid reabsorption occurs (Wong and Yeung, 1978), creating a hypertonic luminal fluid (Levine and Marsh, 1977) because of the increase of sperm concentration.

The first AQP described in epididymis was AQP9, which was present in the stereocilia of principal cells (not basal cells) of rat (Badran and Hermo, 2002) and human (Pastor-Soler *et al.,* 2001), suggesting that this AQP may be involved the electrolyte movement across cell membranes associated to the fast size increasing of spermatocytes. Further studies also demonstrated the presence of AQP2, 5, 7 and 11 mRNA in the rat

epididymal epithelium (Da Silva *et al.*, 2006). In addition, Hermo *et al.* (2004) described that AQP3 takes an exclusively location in the basal cells of the epididymis.

When AQP1 was studied, no expression was detected at epididymal epithelial level. However, it is expressed in the endothelia of vascular regions as well as AQP10 does (Badran and Hermo, 2002; Hermo *et al.*, 2004).

AQP8 expression at epididymal level is controversial. Some studies demonstrate its presence in the basal cells of epididymis (Elkjaer *et al.*, 2001) and other studies did not detect any protein at the same level (Da Silva *et al.*, 2006).

Moreover, AQP5 and AQP9 share location in the apical membrane of principal cells of corpus and cauda membrane, while AQP2 in the distal region of young rats but not in the adult ones maybe because a post-transcriptional mechanism on its regulation.

At the vas deferens level, AQP1 was found to be present in the rat epithelial cells of the ampulla (Brown *et al.*, 1993) and AQP2 in the epithelial cells of the middle vas deferens (Nelson *et al.*, 1998) at the apical plasma membrane where AQP1 was strongly expressed. Unlike these AQPs, AQP9 is the only AQP detected in the whole length of the vas deferens (Pastor-Soler *et al.*, 2001).

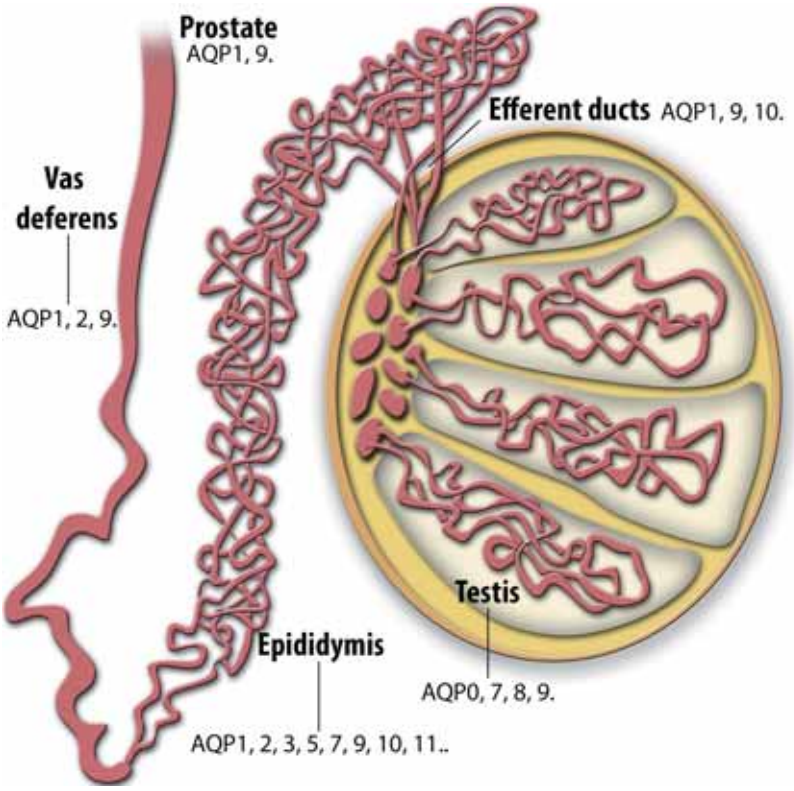


Fig 7 Expression of different AQPs in male reproductive tract of mammal species.

2bb.- AQPs in the female reproductive tract

To date, eleven AQPs (AQP1- 9, 11 and 12) have been described to play a role in water movement between capillaries, interstitial and luminal compartments in the female reproductive tract (Huang *et al.,* 2006). As previously mentioned, expression of some these AQPs, specifically AQP1, 2, 5 and 9, are regulated by steroid sex hormones such as progesterone (P_4) and estradiol (E2) (Jablonski *et al.,* 2003; He *et al.,* 2006; Lindsay *et al.,* 2006). This fact provides an evidence of the role that AQPs play in the reproductive physiology by creating edema and glandular secretion in the uterus (Jablonski *et al.,* 2003; He *et al.,* 2006; Lindsay *et al.,* 2006). The uterine distribution of AQP1, 2, 3 and 8 isoforms suggests that they participate in water flow during uterine imbibition (Braés *et al.,* 2006; Aralla *et al.,* 2009; kowronski *et al.,* 2010; Klein *et al.,* 2013).

It is well known that of all the solutes that are needed during pregnancy for fetal growth, water is without any doubt the most important (Morris *et al.,* 1994). As water requirements increase during pregnancy due to an increase in fetal weight (Damiano, 2011), it seems logical to think that AQPs might be involved in the maintenance of pregnancy and fetal development. Many authors (Mann *et al.,* 2005; Shioji *et al.,* 2006; Beall *et al.,* 2007) have described the involvement of AQPs in the amniotic fluid volume control because of its location in the different fetal membranes. This fact results on a maternal-fetal water flux critical for normal fetal growth (Beall *et al.,* 2007).

As it has been previously stated, eleven AQPs have been described in the reproductive tract, in addition to other locations with several functions.

2bc.- Role of aquaporins on female reproduction

- **Uterine imbibition**

Uterine imbibition is considered a special type of fluid diffusion that undergoes during estrus cycle and pregnancy of mammalian uterus. This kind of diffusion has the characteristic of absorbing water within solids-colloids causing a huge increase of uterine volume. As it has been previously stated, steroid hormones provoke that the uterus undergoes through several changes such as hyperemia, higher capillary permeability, edema and transcellular and intraluminal fluid movement (Finlay *et al.,*

1983; Maier and Kuslis 1988; Cullilan-Bove and Koos, 1993; Okada *et al.*, 2001). Clemenston *et al.* (1997) described that estradiol-17α induces the secretion of water and other substances, such as sodium and potassium, into the lumen of uterine horns in ovariectomized rats, while progesterone was the responsible for the reabsorption of these substances.

In order to describe the mechanisms that balance the water flow within the uterus, Jablonski *et al.* (2003) studied the expression and function of some AQPs in the ovariectomized mouse uterus treated with serum steroid hormones. The study describes that AQP1, 2, 3 and 8 might participate in water movement during uterine imbibition. Myometrial AQP1 is slightly regulated by ovarian steroid hormones (Jablonski *et al.*, 2003). AQP1 has been also described in vaginal smooth muscle (Ganon *et al.*, 2000), which suggests that vagina is an organ with rapid flow of water either out or into the muscular cells may occur. AQP2 was absent in animals treated with control vehicles but it was found to be strongly regulated by estrogen in epithelial cells and myometrium of the uterus. AQP3, as it happens with AQP2, was also detected in uterine epithelial cells, suggesting a contribution to water flow into the uterine lumen. In addition, He *et al.* (2006) described that human endometrial AQP2 was menstrual cycle-dependent. During mid-secretory phase, expression of AQP2 is increased and shows a positive correlation with progesterone and estrogens. AQP8 was found also in the myometrium and in the stroma. This AQP moves the water from the myometrium to the lumen, protecting the myometrial layer from edema (Jablonsky *et al.*, 2003).

On the other hand, Lindsay and Murphy (2006) described that progesterone up-regulates AQP5 in the rat uterus.

- **Oocyte transport and oviductal fluid balance**

The mechanisms that control the oocyte transport across the isthmus to the uterus have been described to be the result of muscle contractions followed by edema and vascular distention (Pauerstein and eddy, 1979; Johns *et al.*, 1982; Verco, 1994). Abrami *et al.* (1995) described AQP1 to be located in the longitudinal myometrial layer and the inner myocytes of the circular muscular layer of the myosalpinx of the frog oviduct and suggested that this AQP could regulate the ovum transport by altering the luminal diameter and increasing water movement into the smooth muscle cells. Also AQP5, 8 and 9 have been described to be present in the rat oviduct epithelial cells, suggesting

that this location was involved in the production of oviductal fluid which, along with steroid hormones, may regulate fertilization and early embryo development (Branes *et al.*, 2005).

- **Follicle maturation and oocyte cryopreservation**

Antral expansion of follicles requires a huge and quick massive transport of water under gonadotropin stimulation. AQP7, 8 and 9 are expressed in rat granulosa cells and are supposed to be involved in the water movement into the antral cavity of follicles (Mc Connell *et al.*, 2002). In addition to this three AQPs (AQP 7, 8, 9), AQP3 and 7, which are expressed in mouse oocytes (Edashige *et al.*, 2000; Meng *et al.*, 2005), can meet this need. Moreover, Thoroddsen *et al.* (2011) described the expression of AQP2 and 3 in human pre-ovulatory follicle and in theca and granulose cells during ovulation, with an acute increase of expression at the beginning of ovulation suggesting an implication in the follicular rupture.

The presence of AQPs in oocytes could lead to improve the methods for a successful cryopreservation of these germ cells. The damage that oocytes undergo during cryopreservation because of the toxicity of cryoprotectants (Hong *et al.*, 1999) may be attributed to AQP3 and 7 that are not only water transporters, but also other solutes transporters as glycerol (Agre *et al.*, 2002). Edashire *et al.* (2003) described that the induced expression of AQP3 in mouse oocytes improved water and glycerol flow across the oocyte membranes, improving its survival. In addition, controlled ovarian stimulation results in a decrease of AQP3 expression mRNA in mouse metaphase II oocytes, suggesting its low survival after cryopreservation (Meng *et al.*, 2005).

- **Blastocyst formation:**

A blastocyst consists in a spherical shell of epithelial cells (trophoectoderm) that surrounds a fluid-filled cavity and an inner cell mass, progenitors of the future embryo (Watson, 1992). The development of the trophoectoderm begins with the adhesion of the morula cells and the inflow of water within the morula. This process ends with the cavitation of the embryo, the formation of the extraembryonic membranes and the beginning of the contact with the endometrium in invasive species (Senger, 2003).

Offenberg *et al*, (2000) described that mRNA of periimplantational mouse embryos encodes seven AQPs (AQP1, 3, 5, 6, 7 and 9), which are expressed from the one cell

embryos stage until the blastocyst stage. In addition, AQP8 was found to be present in morula and blastocyst phases. Moreover, Barcroft *et al.* (2003) described that these AQPs present in the murine embryos modulate water movement across this epithelium following a hyperosmotic gradient.

- **Embryo implantation**

Richard *et al.* (2003) studied AQP1, 4, 5 and 8 expression during the peri-implantational period. The authors hypothesize that the combination of these specific isoforms are responsible of the uterine edema that occurs during implantation. It was described that AQP1 was expressed in the myometrial smooth muscle and can be up-regulated by estrogen in the vessels of the uterus, supporting the hypothesis that this AQP may be involved in the creation of edema. AQP4 is expressed in the luminal epithelia and is up-regulated at the suitable time for mating or insemination. AQP5 is exclusively expressed in the glandular epithelia after blastocyst attachment occurs under the influence of estrogen.

Lindsay and Murphy (2004) described that the increase of AQP1 expression in the mesometrial muscle contributes to the antimesometrial positioning of the embryo in the uterine lumen. The same authors described that AQP5 reorganize its expression from a completely cytoplasmic location during the first days of pregnancy, to a predominant apical plasma membrane organization at the time of implantation in the rat uterus. This fact would explain the pathway for uterine luminal fluid reduction at the time of implantation, just in collaboration with AQP1 and AQP4.

In the same way, He *et al.* (2006) described a high expression of AQP2 at the mid-secretory phase of human endometrium suggesting a role for embryo receptivity. All these facts suggest that AQPs might regulate tissue fluid balance during implantation.

- **Amniotic fluid reabsorption**

It is well known that amniotic fluid provides a fluid-filled compartment essential for normal fetal growth and development. To date, six AQPs (AQP1, 3, 5, 8, 9 and 11) among the 13 identified in mammals have been described in mammalian chorionic membranes and placenta.

AQP1 expression was found in murine syncytiotrophoblasts and chorionic endothelium (Johnston *et al.*, 2000) and in human chorion and amnion cytotrophoblasts (Prat *et al.*,

2012). AQP1 was also detected in placental blood vessels in human beings (Zhu *et al.*, 2009).

AQP3 was described by Johnston *et al.* (2000) to be expressed in the ovine (Johnston *et al.*, 2000) and human (Mann *et al.*, 2002) chorion and placenta but without expression in the amniotic epithelium. AQP3 is apparently the most highly AQP expressed in the placenta with a similar expression for AQP8 in the ovine trophoblastic and epithelial cells (Liu *et al.*, 2004). AQP3 is also expressed in the placental labyrinth, amnion and yolk sac of canines (Aralla *et al.*, 2012).

De Falco *et al.* (2007) found aquaporin 4 expression not only in the human syncytiotrophoblast from the first to the third trimester of pregnancy, but also in the endothelial cells and stroma of placental villi. Also, Escobar *et al.* (2012) described AQP4 and 5 expression in human chorionic villi samples from 10th to 14th week in normal pregnancies. Moreover, Aralla *et al.*, (2012) described a specific staining for aquaporin 5 in amniocytes and columnar cells of allantochorion during canine pregnancy.

AQP8 was first described to be present in human epithelial cells of chorion and amnion and in the syncytiotrophoblasts of human placenta (Wang *et al.*, 2001). Another study demonstrated that AQP8 is strongly expressed in the rat placenta, making it permeable to water and urea but not to glycerol (Ma *et al.*, 1997). Similar results were found in canine (Aralla *et al.*, 2012) and mice placenta (Kobayashi *et al.*, 2010).

Damiano *et al.* (2001) described by RT-PCR, immunobloting and immunochemistry the presence of AQP9 in the apical membranes of syncytiotrophoblasts of human term placenta. Further studies realized by Wang *et al.* (2005) reported AQP9 mRNA expression in ovine amnion and allantois indicating to be a major water channel for intramembranous amniotic fluid reabsorption.

The study of Escobar *et al.* (2012) revealed for first time the expression of AQP11 in the chorionic villi of human placenta between the 10^{th} and 14^{th} week of pregnancy. Moreover, Prat *et al.* (2012) also described AQP1 mRNA and protein in human chorion and amnion.

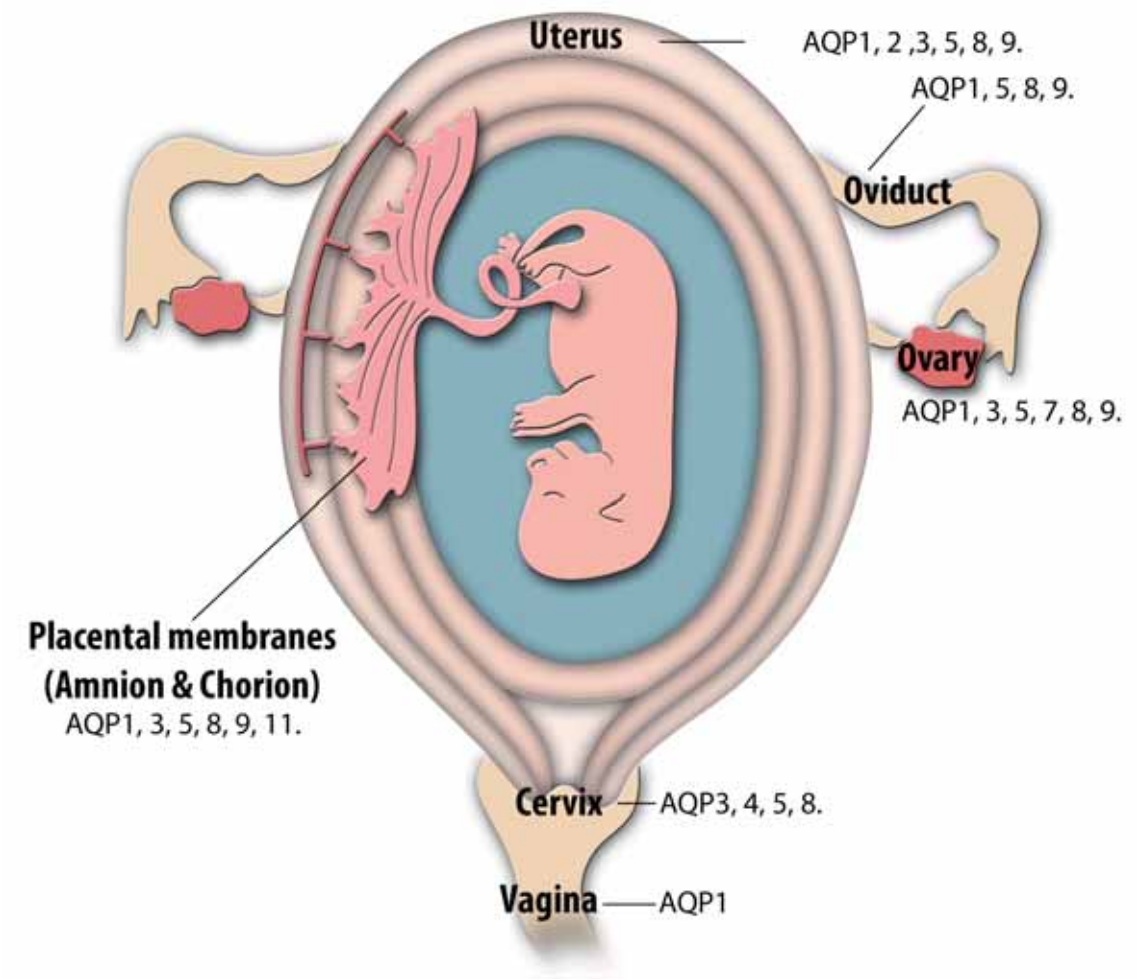


Fig. 8 Expression of different AQPs in female reproductive tract of mammals.

3.- GLUCOSE TRANSPORT

Glucose is the main source of energy for eukaryotic cells (Bell *et al.*,1990). Its oxidation in aerobic and anaerobic conditions supplies ATP to cells. The plasma membrane is not permeable to polar solutes as glucose. For this reason, the cellular catchment of this nutrient is mediated by protein transporters located in the plasma membrane that modulate their pass the lipid bilayer membrane. These transporters were described at the early '50s by observing the dynamics of glucose uptake in erythrocytes (Widdas, 1952; Wheeler and Hinkle, 1981). In mammals, two families of glucose transporters have been described: 1) Na^+-glucose cotransporters (SGLTs) and 2) facilitated diffusion glucose transporters (GLUTs).

SGLTs are mainly located in the apical membranes of the kidney and intestinal epithelial cells (Turner and Silverman, 1977; Shirazi-Beechey *et al.,* 1991). Their function is to modulate the active transport of sugar, mainly glucose, through the cell membrane by facilitated diffusion (Sheepers *et al.,* 2004).

Because glucose is a vital source of energy, GLUTs are present in all tissues. They are considered as passive transporters that allow the transition of substrates through the plasma membrane following the concentration degree (Mueckler *et al.,* 1994). GLUTs are considered a family of integral membrane proteins encoded by the SCL2 gene involved in the hexoses transport (Uldry *et al.,* 2004). Muekler *et al.* (1985) described for first time the sequence and structure of this glucose transporter. GLUTs were described as a 55kDa protein with 12 α-helix domains with an oligosaccharide binding site and the N and C-terminus in the cytoplasmic domain (Fig. 7).

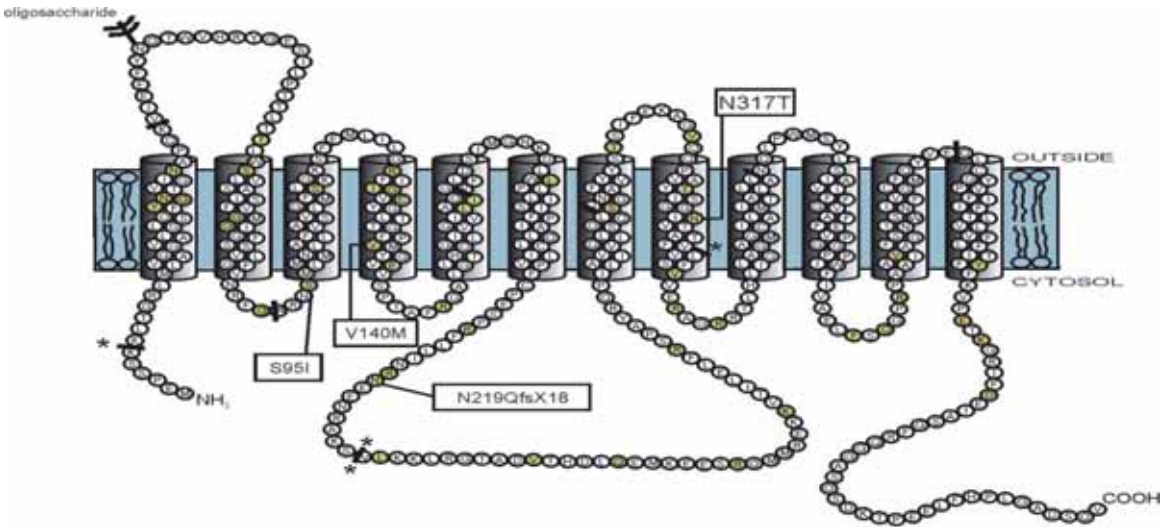


Fig.9 Molecular structure of GLUT1 (Mueckler *et al.,* 1985)

3a.- Molecullar structure and classes of glucose transporters.

To date, 14 GLUTs have been described. All of them have in common that are composed by approximately ~500 amino acid residues and have been classified into three different classes according to their sequence, tissue distribution and hexose specificity (Joost & Thorens 2001; Sheepers *et al.,* 2004). Tissue expression for the different GLUTs is summarized in Fig. 8.

Class I facilitative transporters

GLUTs included in class I are the most studied transporters, composed by a similar structure, tissue distribution and hexose family. GLUTs 1, 2, 3, 4 and 14 are included in this class.

GLUT1 was the first glucose transporter described because of its highly expression (5% of the membrane) in human red blood cells (Muekler *et al.*, 1985). It is located almost in every body tissue and even if it is low expressed, can be found co-expressed with a more highly specific transporter (Muekler, 1994). It has been found to be present in every mouse embryo tissue from the oocyte stage on (Muekler, 1994). Other important locations of GLUT1 were is highly expressed are the endothelial and epithelial cells that constitute blood-tissue barriers as blood-brain barrier (Kalaria *et al.*, 1988), blood-nerve (Floehner *et al.*, 1988), blood-eye (Harik *et al.*, 1990) and placental syncytiotrophoblast (Barros *et al.*, 1992), suggesting that GLUT1 might play an important role in glucose transfer across these barriers.

GLUT2 has been reported to be very important in diabetes (Shepard, 2004), showing that knockout mice develop earlier diabetes. It is expressed in pancreatic β-cells, liver and kidney. In addition, in epithelial cells, GLUT2 is expressed only in the basolateral membrane, transporting glucose from the intracellular compartment to the blood circulation, but, at the other side of the cell, exists a SGLT transporter that acts by up-taking the glucose from the interstitial lumen (Sheepers *et al.*, 2004; Urly and Thorens, 2004).

GLUT3 has been described to be the neuronal glucose transporter (Kayano *et al.*, 1988). GLUT3 has higher affinity for glucose than GLUT1 because of its low Km rate (Muekler, 1994). When GLUT3 was studied in other cell types, its expression was observed in sperm, preimplantation embryos, white blood cells, placenta, carcinoma cell lines, intestine and skeletal muscle; all of them considered cells with high requirements of glucose (Uldry and Thorens, 2004). Nowadays, different studies support the hypothesis that GLUT3 is present in all cell processes (Simpson *et al.*, 2008).

GLUT4 has a high affinity for glucose and it is found in insulin-responsive tissues (Fukumoto *et al.*, 1989), showing higher expression in the adipose tissue than in the skeletal muscle and co-expressed with GLUT1 (Muekler, 1994). GLUT4 reduces the

rising of postpandrial plasma glucose levels (Muekler, 1994). Insulin provokes GLUT4 translocation from the intracellular compartment to the plasma membrane increasing 10-20 fold the glucose transport (Shepherd & Kann, 1999; Bryant *et al.*, 2002).

Lastly, GLUT14 has been described to be a mutation of GLUT3 gene sharing a 95% of its aminoacidic structure and specifically located in testis (Wu and Freeze, 2002).

Class II facilitative transporters

This class is headed by the GLUT5 fructose transporter. In addition, GLUT7 and 9 (with no specific function) and GLUT11 are included in this classification.

GLUT5 is expressed predominantly in liver, testis and intestine, and shows a very low affinity for glucose but a good fructose transport activity (Kayano *et al.*, 1990; Burant *et al.*, 1992). GLUT5 seems not to respond to insulin stimulus (Muekler 1994) and is not inhibited by cytochalasin B as class I facilitative transporters do (Uldry and Thorens, 2004).

GLUT7 specific locations are currently unknown but it has been mapped to chromosome 1 (Joost and Thorens, 2001) and has a high activity to transport glucose and fructose (Li *et al.*, 2004).

GLUT9 has been detected to be expressed in liver and kidney as a fructose transporter. Carayannopoulos *et al.* (2004) suggested that because of the low levels of mRNA found in placenta, GLUT9 plays an important role in preimplantation.

On the other hand, GLUT11 has been described to have two variants composed by a different number of amino acids and with different locations (Doege *et al.*, 2001; Sasaki *et al.*, 2001). GLUT11 has a very low affinity for glucose but competes for fructose in both isoforms, taking into mind that it may be the main substrate transported by GLUT11 (Doege *et al.*, 2001).

Class III facilitative transporters

The class III transporters are the most different of this family. They are characterized by the presence a short extracellular domain in loop 1 and the absence of a glicosilation motif transposed in loop 9 (Joost and Thorens, 2002). Joost and Thorens (2002)

described that this class is the most ancient phylogenetically because they share many characteristics with bacteria, yeast and *Drosophila*. On the other side, mammalians request for glucose are covered by class I and II facilitative transporters. Class III includes five isoforms (GLUT6, 8, 10, 12 and 13 or HMIT).

GLUT6 has a very low affinity for glucose and it is distributed in brain, spleen and periphery leukocytes (Uldry and Thorens, 2004).

GLUT8 have a high affinity for glucose but its activity can be interfered by mannose and fructose suggesting a poli-hexose transport (Doege *et al.*, 2000b). Together with GLUT6 is retained in intracellular structures and the external stimulus for its exposure have not been described yet (Lisinski *et al.*, 2001).

GLUT10 has been related to diabetes type II because of its location at the insulin sensitive tissues as skeletal muscle, liver and pancreas (Dawson *et al.*, 2001). Moreover it has been described in embryonic brain and liver and in adult skeletal muscle (Uldry and Thorens, 2004).

GLUT12 has been described to be a glucose transporter in heart and prostate and is inhibited by mannose and fructose (Rogers *et al.*, 2002; Sheepers *et al.*, 2004).
Finally, GLUT13 (named as HMIT) is expressed especially in the brain and is considered an H^+-myoinositol syntransporter and lacks any sugar transport activity (Uldry *et al.*, 2001).

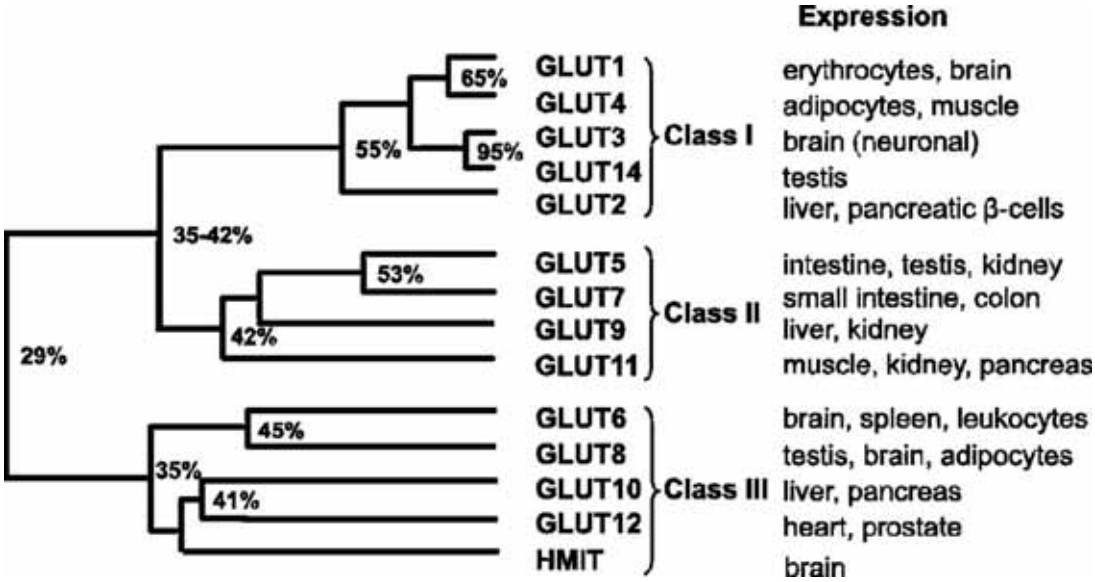


Fig. 10 GLUT families, classes division and expression on different tissues (Sheepers *et al.*, 2004).

3b.- Glucose transport in reproductive physiology

3ba.- Glucose transporters in male genital tract and sperm

Burant and Davidson (1994) described the expression of GLUTs1 and3 in rat testis and also GLUT5 in human testis. GLUT3 and 5 were identified in the juxtaposed cells of the seminiferous tubes. GLUT1 was expressed in the Sertoli cells (Burant and Davidson, 1994). Kim and Moley (2007) described that GLUT8 and 9 were present in the intraseminiferous tubes cells and also in Sertoli cells from mouse testis, suggesting that glucose uptake at seminiferous tubes level may affect to spermatogenesis, steroidogenesis and normal function of mouse sperm. GLUT8 also shows a positive correlation with luteinizing hormone (LH) increasing the testosterone production (Banerjee *et al.*, 2014).

The first report of sugar transporters in sperm cells was from Burant *et al.*, (1992), who demonstrated by western blotting technique the presence of a 50kDa band corresponding to GLUT5 in human testis as well as in ejaculated sperm cells. Haber *et al.* (1993) described also the presence of GLUT3 in human and rat testis and sperm cells. Angulo *et al.* (1998) were the first who described the particular location of glucose transporters in human, rat and bull testis and sperm. In human sperm cells,

GLUT1 and GLUT2 were expressed in the acrosome and in the end piece of the tail, GLUT3 was expressed in the mid piece and GLUT5 was detected in the subequatorial region, principal and mid piece. In rat sperm cells, GLUT1 was detected in the apical membrane of the head and in the end piece of the tail, GLUT2 was described in the acrosome, GLUT3 was described in the principal and mid piece and GLUT5 in the head and mid piece. Bull spermatozoa showed a GLUT1 expression in the acrosome and the end piece of the tail and GLUT2, GLUT3 and GLUT5 in the head and mid piece. Moreover, glucose transporters have also been found in dog (Rigau *et al.*, 2002), boar (Medrano *et al.*, 2006), stallion and donkey (Bucci *et al.*, 2011) spermatozoa with same distribution as in rat, human and bull. Moreover, in the mid piece, Kim & Moley (2007) described that GLU8 was located in the outer dens fibers and in the circumference of the spiral mitochondria. In the principal piece, GLUT8 was located in the outer dens fibers. On the other hand, GLUT9 was located in the mid piece, principal piece and in the acrosome of mice sperm.

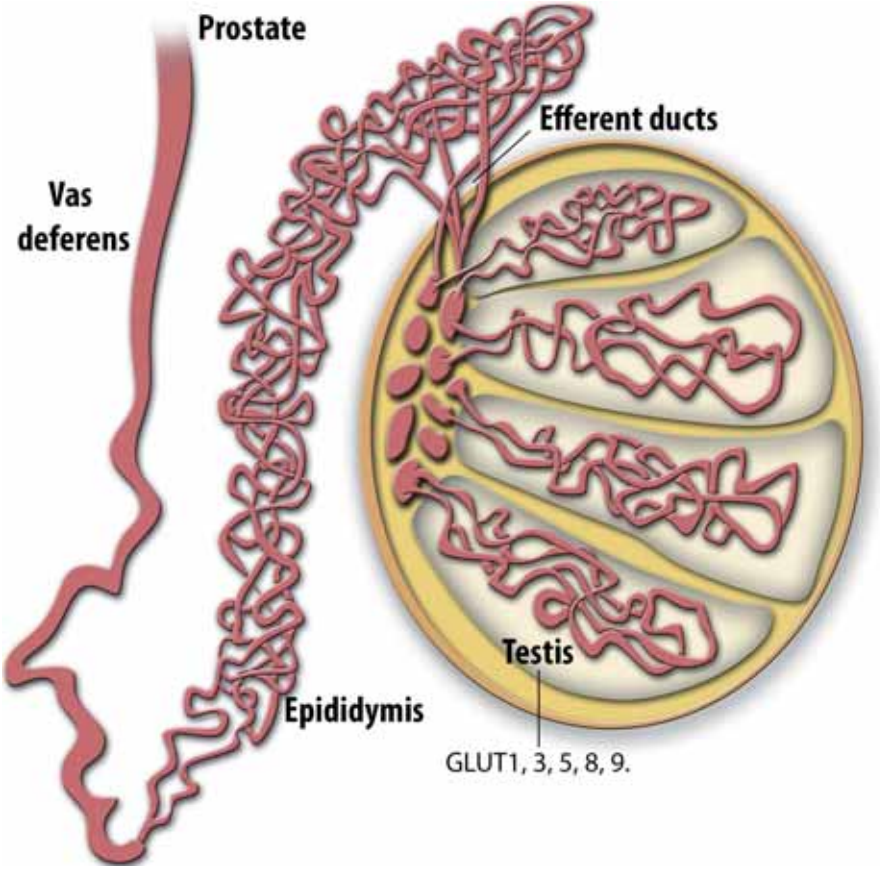


Fig. 11 Expression of different GLUTs in male reproductive tract of mammals

3bb.- Glucose transporters in female reproductive tract

The expression of several GLUTs has been described in mouse and human uterus and some of them have been characterized in terms of possible roles.

3bba.- Expression in the uterine endometrium

GLUT1 was the first to be described in the uterine tissue, specifically in the rat decidua, and was found to be up-regulated during pregnancy, suggesting that it is important for the maintenance of the embryo (Yamaguchi *et al.*, 1996). Moreover, Korgun *et al.* (2001) described its presence in the luminal epithelium, decidua and stroma of the rat uterus. Its expression increased from day 8 of pregnancy on. Frolova and Molley (2011) found that GLUT1 was the most abundant glucose transporter in the mouse uterus, suggesting that may play a role in pregnancy progression. This finding was confirmed by the increase of mRNA expression *in vivo* and *in vitro* of GLUT1 in mouse endometrium during deciduation process (Frolova *et al.,* 2009). Several authors have described an increase of functionality of GLUT1 when ovarian steroid hormones were administrated (Welch and Gorski, 1999; Frolova *et al.,* 2009; Kim and Molley, 2009). These authors also described its presence in the luminal and glandular epithelia of the uterine tissue. Moreover, progesterone administration induces an increase in the plasma membrane expression of GLUT1 while the intracellular membrane fractions remained as previously described (Kim and Molley, 2009).

Von Wolf et al. (2009) described that GLUT1 was up-regulated during the transition of the proliferative phase of the menstrual cycle (E2-dominated) to the secretory phase (P4-mediated). Moreover, during early pregnancy, GLUT1 was also up-regulated. Similar results were found during deciduation in mouse (Frolova & Molley, 2011).

GLUT3 has been described in mouse decidua with a down-regulation during progression of early pregnancy (Yamaguchi *et al.,* 1996). Korgun et al. (2001) confirmed this fact in the rat uterus at day 1 of pregnancy, in addition to an increase of its expression after day 4 of pregnancy. Moreover, a microscope study described that GLUT3 was more abundant in the uterine stroma than in the epithelium. On the other side, no changes in the expression of GLUT3 at endometrium and decidua level of human uterus were observed during menstrual cycle or early pregnancy (von Wolf *et*

al., 2003; Korgun *et al.,* 2005). Moreover, GLUT3 has been seen to be the second and third glucose transporter most present respectively in human and mouse endometrium and decidua (Frolova and Molley, 2011).

GLUT 4 data is very controversial. Von Wolf et al. (2003) excluded GLUT4 from human uterus, but other authors cited its presence in the endometrium (Mozzanega *et al.,* 2004; Kohan *et al.,* 2010). However, GLUT4 immunostaining was only detected at endometrial epithelial cells level (Mozzanega *et al.,* 2004).

Finally, GLUT8 has been found only in the endometrium in all the studied species, and in the case of rat uterus its expression increase during estrus and embryo implantation (Kim and Molley, 2009). Unlike GLUT1 location, GLUT8 is expressed in both apical and basolateral membranes of both glandular and luminal epithelium and not in the cell surface (GLUT1, 3 and 5). Because of an amino-terminal dileucine motif, GLUT8 has an intracellular location (Carayannopoulos *et al.,* 2000). Nevertheless, stimuli for this translocation mechanism have not been yet described.

3bbb.- Glucose transporters in placenta

GLUTs catalyze the hexose transport across many tissues including the placenta (Sciullo *et al.,* 1997). Transplacental passage of glucose is accomplished by facilitated diffusion because of the presence of a sodium-independent transport system of D-glucose in the apical and plasma membrane of the trophoblasts (Desoye *et al.,* 2011). Three GLUTs have been described in the human placenta (GLUT1, 3 and 4). GLUT1 has seen to be expressed in all the placental cells during different stages of human, marmoset monkey and rat placenta (Hahn *et al.,* 1995). Hahn et al. (1998) described that hyperglycemia downregulates human placental GLUT1 and induces to its endocytosis from the plasma membrane into the interior of the cell (Hahn *et al.,* 2000). Moreover, GLUT1 has been described in carnivorous placenta of cats, minks and dog (Wooding *et al.,* 2007), showing a strong expression in the basolateral membranes of the trophoblasts. On the other side, GLUT3 and 4 are expressed in specific locations, giving to these glucose transporters a specialized function. It has been described that GLUT3 is expressed in stromal cells and endothelium of feto-placental vessels of human term placenta (Hahn *et al.,* 2001). Brown et al. (2011) described that its expression decreases through trimesters in human placenta, suggesting that GLUT3 is

of greater importance for glucose uptake during early pregnancy. GLUT3 is also expressed in amnion, chorion, yolk sac and in the poorly differentiated placental trophoblasts (Hahn *et al.,* 2001). Surprisingly, GLUT4 is expressed in the stromal cells of human placenta (Xing *et al.,* 1998). Its function has been discussed because of its insulin-dependent characteristic. Nevertheless, it has been suggested that its function may be focused on the insulin regulation of the glucose uptake from the fetal circulation to the stromal cells to cover the requirements (Desoye and Shafrir, 1994). Recently, GLUT8 has been found in the decidual cells of rat and human placenta (Carayannopoulus *et al.,* 2000). Finally, GLUT12 has been described in human trophoblasts during the first trimester of pregnancy and in vascular smooth muscle and stromal cells at term pregnancy (Gude *et al.,* 2002), suggesting that GLUT12 is vital to trophoblast function at early pregnancy.

3bbc.- Glucose transporters in embryo

In 1991, Hogan et al. (1991) described for first time the expression of GLUT1 and GLUT2 in mouse preimplantational embryos. GLUT1 function in embryo has been described to be crucial for glucose uptake from 1-cell- embryo on. It is at 8-cell embryo stage when GLUT2 starts to express. The glucose up-take from 8- embryo and further can be carried to term by these two transporters (Gardner and Leese, 1988). In addition, GLUT2 expression during blastocyst stage was restricted to the basal trophoectoderm in direct contact with the blastocele cavity, while GLUT1 was expressed at the basolateral, apical and intercellular junctions between cells (Hogan *et al.,* 1991). Seven years later, Pantaleon et al. (1997) described for first time the expression of mRNA GLUT3 in murine embryos at late four-cell stage and GLUT3 protein at eight-cell morula stage. Its location during the morula stage was basically in the apical surface of the polarized outer cells and, during blastocyst stage, in the apical surface of the trophectoderm, suggesting that GLUT3 is a critical transporter of transplacental glucose necessary for fetal growth.

GLUT8 has also been described to have an intracellular location in murine embryos. Insulin or insulin growth factor-1 (IGF-1) translocate its location to the plasma membrane, increasing the glucose uptake (Carayannopoulus *et al.,* 2000). Moreover, it

has been described that intracellular retention of GLUT8 increases the embryo apoptosis decreasing its survival (Pinto *et al.*, 2002).

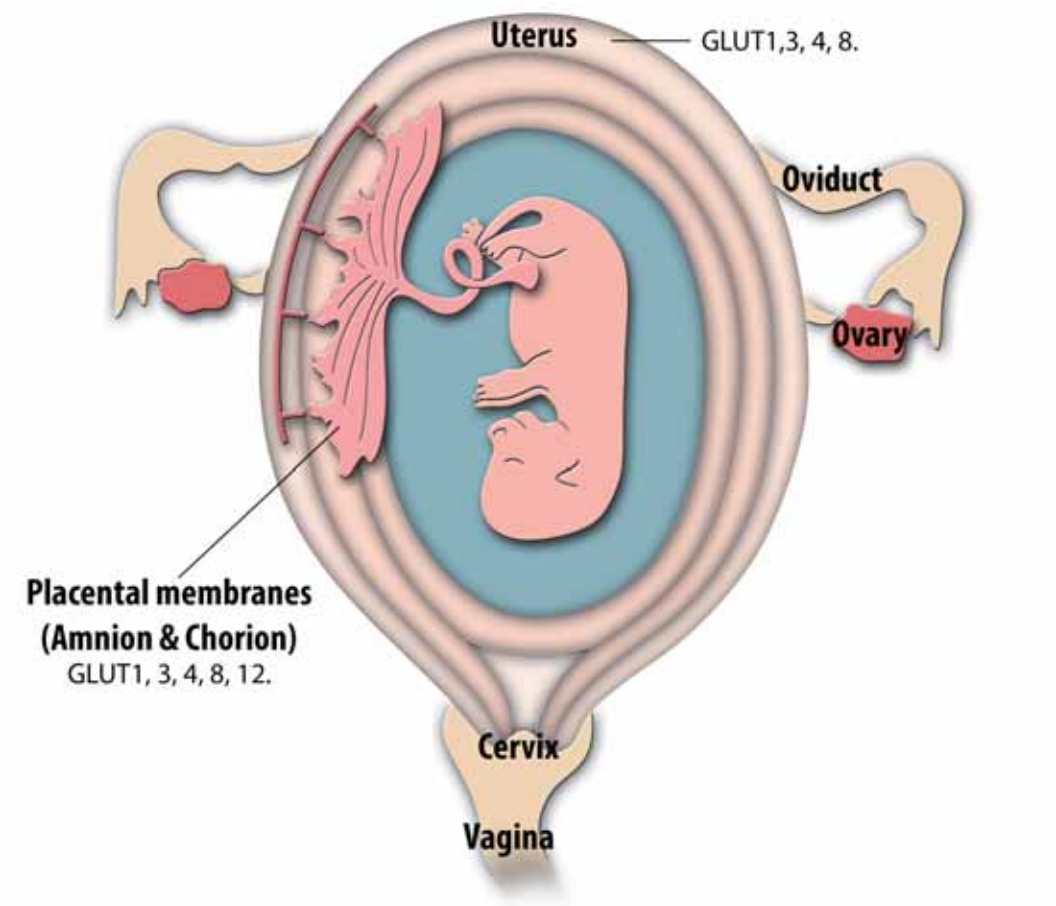


Fig. 12 Expression of different GLUTs in female reproductive tract in mammals.

Aims of the study

This PhD study was aimed at a better understanding of transplacental transport in queen reproduction, by deepening the knowledge in those species in which studies on aquaporins and glucose transporters have been already performed and furnishing new information in a new species which these arguments have not been approached yet.

Based on the described literature in the previous section, the objectives of the present PhD thesis were divided in three main blocks, with every individual block corresponding to an original article:

Article 1:

1. To determine the presence and the specific location of AQP-2 in the endometrium of pregnant and non-pregnant queens and placental transfer zone of pregnant queens at different pregnancy stages.
2. To determine the possible correlation between endometrial and placental AQP-2 expression with serum progesterone concentration.
3. To determine variations of aquaporin-2 activity mediated by phosphorylation of tyrosine residues.

Article 2:

1. To determine the presence, the specific location and the possible correlation between aquaporins-1, -3 and -8 in the endometrium of pregnant and non-pregnant queens and placental transfer zone of pregnant queens at different pregnancy stages.
2. To determine the possible correlation between aquaporins 1-, 3, and-8 and serum progesterone levels.

Article 3:

1. To determine the expression, the specific location and the possible correlation between glucose transporters GLUT1 and GLUT3 in the endometrium of pregnant and non-pregnant queens and placental transfer zone of pregnant queens at different pregnancy stages.

   To determine the possible correlation between GLUT1 and GLUT3 with serum progesterone levels

Article 1: Aquaporin-1, -3 and -8 in queen reproductive tract may handle water transport in queen reproductive tract and placental transference zone. Evaluation at different phases of the sexual cycle and pregnancy stages.

Elsevier Editorial System(tm) for Theriogenology

Manuscript Draft

Manuscript Number:

Title: Aquaporin-1, -3 and -8 in queen reproductive tract may handle water transport on queen reproductive tract and placental transfer zone. Evaluation at different phases of the sexual cycle and pregnancy stages

Article Type: Original Research Article

Keywords: Aquaporin, queen, placental transference zone, endometrium, pregnancy

Corresponding Author: Dr. Maria Montserrat Rivera del Alamo, PhD, ECAR Diplomate

Corresponding Author's Institution: Autonomous University of Barcelona

First Author: Lluís Ferré-Dolcet

Order of Authors: Lluís Ferré-Dolcet; Marc Yeste, PhD; Meritxell Vendrell; Teresa Rigau, PhD, ECAR Diplomate; Joan Enric Rodríguez-Gil, PhD, ECAR diplomate; Maria Montserrat Rivera del Alamo, PhD, ECAR Diplomate

Abstract: Aquaporins (AQPs) are known to be involved in water flow across tissues and are widely described in reproductive functions. The present study was focused on the determination of the expression and location of AQPs1, 3 and 8 in endometrium and placental transference zone of non-pregnant and pregnant queens at different sexual and gestational stages. Queens were split in six different groups: non-pregnant with low levels of serum progesterone, non-pregnant with high levels of serum progesterone, 30, 40, 50 and 60 days of pregnancy. Samples from uterus and placental transference zone were evaluated by immunoblotting and immunohistochemistry techniques. Immunoblotting identified 29-32 kDa bands corresponding to the different AQPs in every stage evaluated. No significant changes in the expression of these AQPs in the different stages were found. On the other hand, AQP1 showed a negative correlation with serum progesterone levels while have a positive correlation with AQP3 expression. Immunohistochemistry demonstrated the presence of AQP1, 3 and 8 in glandular and laminar epithelia and myometrium. AQP1 was also present in the vascular endothelia from the endometrium. AQP3 and 8 were present in the chorionic structures, while AQP1 was absent. AQP8 showed changes in distribution related with serum progesterone concentration. When progesterone was above 2 ng/ml, AQP8 was distributed in the cell membrane and cytoplasm, while low levels of progesterone under 1 ng/ml kept AQP8 located only in the cell membrane. In conclusion, AQPs1, 3 and 8 have been described for the first time in queen reproductive tract. Furthermore, our results suggest that AQPs 1, 3 and 8 may contribute in the regulation of water metabolism during queen pregnancy.

Aquaporin-1, -3 and -8 in queen reproductive tract may handle water transport on queen reproductive tract and placental transfer zone. Evaluation at different phases of the sexual cycle and pregnancy stages

L. Ferré-Dolcet[1], M. Yeste[2], M. Vendrell[1], T. Rigau[1,] JE. Rodríguez-Gil[1*] MM. Rivera del Alamo[1*]

[1]*Animal Medicine and Surgery Department, Veterinary College, Universitat Autònoma de Barcelona, Bellaterra, 08193, Spain*

[2]*Department of Biology, University of Girona, Girona, E-17071 Girona, Spain*

**Corresponding author: VO-229, Departament de Medicina i Cirurgia Animals, Facultat de veterinària, Edifici V, Campus Universitari, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. Tel.: +34 935811045.*

E-mail addresses: mariamontserrat.rivera@uab.cat juanenrique.rodriguez@uab.cat

Abstract

Aquaporins (AQPs) are known to be involved in water flow across tissues and are widely described in reproductive functions. The present study was focused on the determination of the expression and location of AQPs1, 3 and 8 in endometrium and placental transference zone of non-pregnant and pregnant queens at different sexual and gestational stages. Queens were split in six different groups: non-pregnant with low levels of serum progesterone, non-pregnant with high levels of serum progesterone, 30, 40, 50 and 60 days of pregnancy. Samples from uterus and placental transference zone were evaluated by immunoblotting and immunohistochemistry techniques. Immunoblotting identified 29-32 kDa bands corresponding to the different AQPs in every stage evaluated. No significant changes in the expression of these AQPs in the different stages were found. On the other hand, AQP1 showed a negative correlation with serum progesterone levels while have a positive correlation with AQP3 expression. Immunohistochemistry demonstrated the presence of AQP1, 3 and 8 in glandular and laminar epithelia and myometrium. AQP1 was also present in the vascular endothelia from the endometrium. AQP3 and 8 were present in the chorionic structures, while AQP1 was absent. AQP8 showed changes in distribution related with serum progesterone concentration. When progesterone was above 2 ng/ml, AQP8 was distributed in the cell membrane and cytoplasm, while low levels of progesterone under 1 ng/ml kept AQP8 located only in the cell membrane. In conclusion, AQPs1, 3 and 8 have been described for the first time in queen reproductive tract. Furthermore, our results suggest that AQPs 1, 3 and 8 may contribute in the regulation of water metabolism during queen pregnancy.

42 Keywords: Aquaporin, queen, placental transference zone, endometrium, pregnancy.

43

Introduction

Fluid balance is known to be very important in the female reproductive tract [1]. Hormonal stimuli contribute to uterine edema in the cycling endometrium [2] creating an appropriate environment for reproductive functions. At the time of embryo implantation and during pregnancy, endometrial vascularization is increased due to the preparation of the tissue for fetal implantation and development [3]. In addition to the increase of the endometrial vascularization, water requirements also increase due to an exponential growth in the fetal weight [4]. Mammal uterus contains endometrial glands that produce secretion to facilitate the process of embryo implantation and to maintain the appropriate environment for the conceptus during pregnancy [5]. On the other hand, the outer layer of the fetal placenta, the chorion, invades the endometrium for the forward nutrition of the embryo [6].

Aquaporins (AQPs) have been widely described in the eukaryotic cell and a review of that particular issue is beyond the aim of this article. Focusing on the reproductive tract, AQP1 was the first to be described, in particular in the human uterus [7]. Since then, several authors have described the presence of these water channels in the female reproductive tract. AQP1 has been found in the muscular layer of the rat oviduct [8], in the endothelial cells of the endometrium and the circular layer of the myometrium of the rat [9], in the pig endothelial cells of uterine blood vessels [10] and in the vascular endothelia, glandular epithelium and myometrial cells of the uterus in the bitch [11].

AQP3 was described for the first time in the basolateral membranes of the cells from the collecting ducts of the kidney [12]. Later on, it has also been described in a large number of extra-renal localizations such as urinary bladder, epithelia,

erythrocytes, leukocytes, skeletal muscle, skin, prostate and placenta [13, 14]. In the female reproductive system, AQP3 is present in the epithelial and columnar cells of the glandular epithelium of the human endometrium [15]. Jablonski et al. [15] described the presence of AQP3 in uterine epithelial cells. According to these results, the presence of AQP3 in the uterine lumen suggests that it may contribute to the water movement into the uterine lumen. In addition to uterus, AQP3 has been also described in the rat placenta [13] and in the ovine trophoblast cells [16].

AQP8 has been widely reported to be present in mouse, rat and porcine testis [17, 18] and it is also strongly expressed in the mouse placenta [19]. The first evidence of AQP8 in human amnion, chorion and placenta was described by Wang et al. [20]. Later, it has been described in the cell cytoplasm in addition to the cell membrane of amniotic and chorionic surfaces [21], suggesting that AQP8 handles water movement in the fetal development.

The aim of this study was to evaluate the expression and localization of AQP1, -3 and -8 in the queen endometrium and placental transfer zone during different stages of the sexual cycle and at different gestational ages. In addition, serum progesterone levels were also monitored in order to establish a possible correlation between the different analyzed AQPs and serum progesterone concentration.

Materials and methods

Collection and processing of samples

A total of thirty-eight queens were included in the present study. The females belonged to a program of sterilization of stray-cats performed at the Surgery Unit, Faculty of Veterinary Medicine, Autonomous University of Barcelona (Bellaterra, Spain).

Included queens had to fulfill the criteria of no sign of current illness and negative tests for feline leukemia (FeLV) and feline immunodeficiency (FIV).

After being anesthetically pre-medicated, a blood sample from the jugular vein was obtained from every queen. Administered pre-medication consisted in ketamine (5 mg/kg, Imalgene 1000, Laboratorios Merial, Barcelona, Spain), buprehorphine (20 μg/kg, Buprecare, Divasa-Farmavic S.A, Gurb, Spain) and midazolam (0.2 mg/kg, Midazolam Normon, Laboratorios Normon S.A., Madrid, Spain) IM. Anesthetic induction was performed with IV propofol (4 mg/kg, Vetofol, Laboratorios Esteve, Barcelona, Spain) and anesthetic status was maintained with 1.5-2% isoflorane (Isoflo, Laboratorios Esteve, Barcelona, Spain) in 2% oxygen.

A routine ovariohysterectomy was performed by midline laparotomy regardless the sexual stage of queens. After surgery, reproductive tracts were evaluated macroscopically and queens were divided into two groups depending on whether they were pregnant or not. Non-pregnant queens were divided into non–ovulated (n=7) and ovulated (n=7) according to serum progesterone levels (see below). Pregnant queens were divided according to their gestational age, which was determined by measuring the diameter of the embryo vesicles [22]. According to this, pregnant queens were divided into 30 (D_{30}; n = 8), 40 (D_{40}; n = 5), 50 (D_{50}; n = 4) and 60 (D_{60}; n = 7) days of pregnancy.

After removing the reproductive tract, full-thickness biopsies from the uterus were obtained from every queen in the study. From the pregnant queens, in addition to uterine biopsies, biopsies from the placental transfer zone were also obtained. Every biopsy of uterus and transference zone was split in two samples. One sample was fixed with 10% paraformaldehyde for 48 hours for immunohistochemistry purposes. The other sample was snap frozen in liquid nitrogen and kept at -80ºC for Western blotting

purposes. In the case of uterine samples for Western blotting analyses, only the endometrium was kept after removing it from the myometrium with a scalpel.

Samples were obtained following the guidelines of the Ethical Committee Animal Care and Research, Autonomous University of Barcelona (CEEAH, code 2939).

Progesterone concentration

Blood samples obtained from the jugular vein were collected in glass tubes and allowed to clot for 10 minutes. After that, samples were centrifuged at 2500x*g* for 10 minutes and were then kept frozen at -20ºC until progesterone analysis was performed (Immulite® 1000, Siemens Healthcare Diagnostics, Cornellà del Llobregat, Spain).

According to serum progesterone levels, non-pregnant queens were divided into two groups: ovulated (progesterone concentration $\geq$ 2 ng/mL) and non-ovulated (progesterone concentration $\leq$ 1 ng/mL) (Fresno et al. 2012).

Western immunoblot analysis

First of all, total protein concentration of samples was determined by the Bradford technique [23]. For that purpose, endometrium and placental transference zone were homogenized in 1 mL of cold protein extraction buffer (50mM TRIS HCl pH 7,4, 1mM EDTA, 10mM EGTA, 25mM DTT, 1.5% Triton-X-100, 1mM PMSF, 10µg/mL Leupeptine, 1mM Ortovanadate, 1mM Benzamidine) with an Ultra-Turmax T25 basic homogenizer (IKA-WERKE, Staufen, Germany). After centrifugation of homogenized samples at 2500x*g* and 4ºC for 5 minutes, supernatants were recovered and kept for the Western blotting analysis.

Proteins were separated by SDS-PAGE electrophoresis in 10% (w/v) acrylamide gels and transferred to nitrocellulose membranes [24]. Proteins transference to the

nitrocellulose membranes was confirmed by 0.1% Ponceau S dye (w/v). Membranes were then blocked against non-specific unions in a 2% BSA (w/v) solution for 1 hour at room temperature and incubated overnight at 4ºC with the corresponding primary antibody. After that, membranes were washed to remove the excess of primary antibody and then exposed during 45 minutes to the corresponding secondary antibody. All antibodies, primary and secondary, were used at a 1:1,000 (v:v) dilution.

Membranes were then exposed to radiograph films to reveal the AQPs bands (28-32 kDa) after 5 minutes of incubation with Inmobilon TM Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, 01821, USA).

After that, nitrocellulose membranes were stripped of with a buffer (10 gr glycine, 1 gr SDS, 10 ml Tween 20, pH 2.2, in 1 L of pure water) in order to remove the specific marking for AQPs. Then, they were re-probed with an anti-mouse α-tubulin antibody (ABR Affinity BioReagents, CO, USA) to verify the same amount of protein was loaded for every sample.

To analyze integral densities of specific AQPs bands, a computer-assisted image analysis system (Multi Gauge v 3.0 software system, Fujifilm, Tokyo, Japan) was used. Densitometry ratios between AQPs and α-tubulin bands were calculated. Three blots (technical replicates) per sample were evaluated. These ratios gave the relative abundance of each AQP.

Immunohistochemistry

Uterine and transference zone biopsies were fixed in 10% paraformaldehyde for 48 hours at 20ºC and then embedded in paraffin blocks. Four μm-thick sections were obtained. For deparaffinization, sections were immersed twice for 5 minutes in Xilene and hydrated through descending concentrations of ethanol (100%, 96% and 70%

169 respectively) and washed twice in PBS (1x) for 5 minutes with slight agitation at room
170 temperature. After that, samples were immersed in a citrate buffer (10mM sodium
171 citrate, 0.05% Tween20, pH=6.0) for 20 minutes at 96ºC for antigen retrieval and then
172 rinsed twice in PBS for 5 minutes. For blocking unspecific antibody reactions, samples
173 were immersed for 1 hour in a 2% BSA solution (w/v) at room temperature. After
174 blocking, samples were incubated overnight at 4ºC with the corresponding primary
175 antibody. All primary antibodies were used at a 1:100 dilution (v/v). Afterwards,
176 sections were washed two times in PBS for 5 minutes, and then incubated for 3 hours at
177 4ºC with the corresponding secondary antibody. All secondary antibodies were used at
178 1:100 dilution (v:v). For cell nuclear staining, samples were incubated with Hoescht-
179 33342 (Thermo Fisher scientific Inc. Waltham, MA, 02451, USA) for 20 minutes.
180 Sections were then sealed with Vectashield HardSet antifade mounting medium (Vector
181 laboratories, Inc. Burlingame, CA, 94010, USA). Evaluation of samples was performed
182 with a Confocal microscope SP5 (Leica Microsystems GmbH, Mannheim, Germany).
183
184 *AQP-antibodies*
185 Primary antibodies for AQPs detection are listed next. For AQP1 detection, a
186 monoclonal anti-rabbit antibody (AbD Serotec, Endeavour House, Langford Lane,
187 Kidlington, OX5 1GE, UK) was used. AQP3 detection was performed with an anti-
188 rabbit polyclonal antibody (Novus Biologicals, Abingdon, OK14, 3NB, UK). Finally,
189 for AQP8 detection, an anti-rabbit polyclonal antibody (Bioss Inc., 500 West
190 Cummings Park, Suite 6500, Woburn, Massachusetts, 01801, USA) was used.
191 Regarding secondary antibodies for immunoblotting, AQP1 was faced to a goat
192 anti-mouse IgG-HRP antibody, while AQP3 and AQP8 were faced to a donkey anti-

rabbit IgG-HRP antibody. All of them were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., 10410 Finnell Street, Dallas, Texas, 75220, USA).

Regarding secondary antibodies for immunohistochemistry, an Alexa Fluor antibody 568-linked anti-mouse antibody was used for AQP1 detection, an anti-rabbit antibody Alexa Fluor 555 antibody for AQP3 and a secondary Alexa Fluor 647-linked anti-rabbit antibody for AQP8. All secondary antibodies were purchased from Thermo Fisher (Invitrogen; Eugene, Oregon, USA).

Statistical analyses

Data were evaluated through a statistical package (IBM SPSS Statistics for Windows 21.0, Chicago, IL, USA) and are shown as mean ± standard error of the mean (SEM). Prior to conducting any statistical test, data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Levene test). In all cases, data matched with parametric assumptions, that is why separate one-way analyses of variance (ANOVA) followed by post-hoc Sidak test were run for the relative abundance of each AQP (AQP1, AQP3 and AQP8). The factor was the queen stage (non-ovulated, ovulated or at different days pregnancy). The same procedure was applied for concentrations of P4 in serum.

Apart from testing differences between groups, Pearson correlations were also calculated between concentrations of P4 in serum and relative abundances of AQP1, AQP3 and AQP8.

For all analyses, the level of significance was set at $P \leq 0.05$.

Results

Western immunoblot analysis

Expression for AQP1, AQP3 and AQP8 was observed in all samples from both endometrium and placental transference zone (Fig 1). When the intensity of the bands was quantified, no statistically significant ($P>0.05$) differences among the groups were observed regardless their gestational age or sexual cycle phase of the queen (Table 1).

On the other hand, a positive and significant correlation ($P<0.05$) between the expression of AQP1 and AQP3 was observed, with an r value of 0.35 (Table 2). Finally, AQP1 showed a statistically significant negative correlation ($P<0.05$) with serum progesterone concentration, with an r value of 0.32 (Table 2).

Immunohistochemistry

The presence of AQP1, -3 and -8 was observed in the cells from both glandular and luminal epithelia in all uterine samples regardless the phase of the cycle or the gestational age (Figs 2, 3, 4). AQP1 and AQP3 were homogeneously distributed in the cytoplasm as well as the plasma membrane. No changes in the localization of these AQPs were observed between the different phases of the sexual cycle or the different gestational ages. However, AQP8 did show a localization change related with serum levels of progesterone. Thus, in queens with low levels of progesterone, AQP8 was mainly found at the plasma membrane (Fig 5). On the contrary, in queens with high levels of progesterone, regardless they were pregnant or not, AQP8 was distributed in both plasma membrane and cytoplasm.

AQP1 and -3 were also located in the myometrial cells from uterine samples in every stage evaluated. In addition, myometrial positive staining to AQP1 was increased in queens with high levels of serum progesterone, regardless if they were pregnant or not. Apart from the epithelial cells, AQP1 was also present in the endometrial vascular endothelia. Myometrial surface did not express AQP8.

Finally, placental transference zone samples expressed AQP3 and AQP8, but not AQP1, in all the gestational ages evaluated. AQP3 and -8 were present in the syncytiotrophoblastic and cytotrophoblastic cells of the chorionic labyrinth with a homogeneous pattern of distribution in both membrane and cell cytoplasm. In addition, cytotrophoblastic cells showed a higher pattern of AQP3-immunoreactive intensity than the syncitiotrophoblastic cells during mid-term pregnancy (Figs 2, 3, 4).

Negative controls for all immunohistochemical analyses were performed in order to modify the technique or replace antibodies against the tested AQPs. All controls confirmed the specificity of our antibodies.

Discussion

As it has been previously stated, fluid balance is important in the reproductive tract [1]. The water movement to the uterus is controlled by different mechanisms, such ovarian estradiol-17β [25], nitric oxide [26] and AQPs [27].

Aquaporins 1, -3 and -8 have been previously described in the female reproductive tract of several mammalian species. The present study describes, for first time, the presence of AQP1, -3 and -8 in the queen uterus and placental transference zone and their possible correlation with serum progesterone levels in non–pregnant queens at different sexual phases and pregnant queens at different gestational ages.

In accordance with other studies [9-11], AQP1 was present in the myometrium, endometrial vascular endothelia and cells from both laminal and glandular epithelia of queen uterus in every stage evaluated. In immunoblotting analyses, intensity of bands showed that AQP1 expression was negatively correlated with serum progesterone levels, suggesting that progesterone could down-regulate its function. These results are in disagreement with previous studies. One study performed in sows showed no

changes in AQP1 myometrial expression with variable concentrations of serum progesterone [10]. On the other hand, Richard et *al.* [27] and Aralla et *al.* [28] described that AQP1 expression was increased in the uterus of bitches with high levels of serum progesterone, suggesting an increase of water flow from the myometrium into the uterine stroma in order to prepare the endometrium for a possible implantation of a conceptus. Thus, according to our results, AQP1 either does not play a role on feline pregnancy development or needs to be blocked for the proper pregnancy development. However, further research is needed to stablish its real role on feline pregnancy.

With regard to immunochemistry results, myometrial cells showed a stronger immunoreactivity staining in non-pregnant queens with high levels of serum progesterone. Previous studies [9, 11, 29, 30] demonstrated an up-regulation of AQP1 at endometrial and myometrial levels with high serum progesterone concentrations. Our immunohistochemistry data are in agreement with those studies. However, we observed a negative correlation between progesterone levels and AQP1 expression evaluated through immunoblotting. This could be explained by the fact that myometrial surface was previously removed with a scalpel in the samples and only endometrium was used for immunoblotting purposes.

In the present study, AQP1 was neither expressed in vascular endothelia of the endometrium nor in the chorionic layer, which suggests that AQP1 is not involved in the water supply at embryonic level. These results differ from other studies that have previously described the presence of AQP1 in the vascular endothelia and trophoblasts of the chorionic labyrinth [28, 31-33]. Thus, this is the first study that describes the absence of AQP1 in the chorionic vascular endothelia in a mammalian species. Taking these results together, the lack of expression in the chorionic cells and the decrease of

expression when serum progesterone increases it could be suggested that AQP1 might not be involved in the water supply to the fetus during queen pregnancy.

Expression for AQP3 in both glandular and luminal epithelium, vascular endothelium and myometrium is observed in every stage evaluated in uterine samples. These results are in concordance with previous studies [14, 15] and suggest that AQP3 may be involved in the uterine imbibition and in the preparation of the uterus for pregnancy. Regarding to the myometrial surface, Wakayama et al. [34] reported a co-expression of AQP3 and AQP4 in skeletal muscle but it is yet to be determined whether they play any function in muscle contraction or metabolism.

The presence of AQP3 in the trophoblastic cells and vascular endothelia of the placental transference zone is in agreement with other studies performed in bitch, human, mouse and sheep [28, 33, 35, 36], suggesting a trophoblastic function of this specific AQP. When expression intensity for AQP3 was evaluated, cytotrophoblastic cells showed higher immunoreactivity than syncitiotrophoblasts from mid-pregnancy on. It is well known that cytotrophoblasts are responsible for the invasion of uterine spiral vessels with the further invasion of the endometrium and the proximal third of the myometrium for the anchoring of the growing fetus and creating the functional placenta [37]. Thus, the increase in AQP3 would suggest that this AQP could be involved in the invasive phase of the endometrium. On the other hand, syncitiotrophoblasts are responsible for the biomolecular interactions between the mother and the fetus by invading the uterine wall and serving as an endocrine organ that produces growth factors for fetal development and some hormones such as progesterone and human chorionic gonadotrophin [28-41]. Regarding to immunoreactivity of AQP3 in syncitiotrophoblasts, it can be suggested that AQP3 plays a constitutional function for solutes transport across the placenta for the proper fetal development.

Concerning to the expression of AQP8 in the female reproductive tract, its expression has been previously described in other species such as mice [15], bitch [11] and human [20]. According to immunohistochemistry results, AQP8 was only expressed in the myometrial surface of non-pregnant queens with high levels of serum progesterone. While Jablonski et al. [15] showed that AQP8 is present in mice myometrial and endometrial stromal cells, which is in disagreement with our results.

Serum progesterone levels induced changes in the localization of AQP8 in the endometrial cells. While queens with high levels of serum progesterone showed expression for AQP8 in both cytoplasm and cell membrane, regardless whether they were pregnant or not, queens with low levels of serum progesterone showed expression for AQP8 only in the cell membrane. This result suggests that progesterone may modulate AQP8 distribution in uterine glandular epithelia. Although previous studies have demonstrated that progesterone is able to induce or modulate changes in the expression of other uterine proteins [42, 43], further research to determine the actual role of progesterone on AQP8 expression is warranted.

As aforementioned, AQP8 is the only AQP able to transport urea in addition to water [20]. Urea is formed because of the nitrogen metabolism that occurs in the liver. During pregnancy, the fetus produces urea that has to be discarded from fetal to the maternal circulation, especially from mid-pregnancy on when the fetus grows exponentially and amniotic fluid volume increases [44, 45]. Thus, as the fetus grows, more urea is produced. The present results suggest that AQP8 expressed in the trophoblastic cells of the chorionic labyrinth could be involved in the urea transport across the fetal membranes. Bearing this in mind, our results are in concordance with other studies [11] that found a high expression of AQP8 in fetal membranes during the last term of pregnancy. Moreover, every sample of transference zone of pregnant

queens showed immunoreactivity in the trophoblastic cells of the chorionic labyrinth in every stage evaluated, bringing to mind the possible role of urea discard across the fetal membranes.

In conclusion, this study determines, for the first time, the presence of AQP1, -3 and -8 in uterine and placental structures of cycling and pregnant queens. According to the present results, it can be hypothesized that a combination among these AQPs may create a functional collaboration in water handle during different sexual stages in both uterus and placental transference zone. However, further studies are needed in order to determine the actual role of these AQPs and combination with other AQPs implied in the water movement for embryo development.

References

[1] Sha XY, Xiong ZF, Liu HS, Di XD, Ma TH. Maternal-fetal fluid balance and aquaporins: from molecule to physiology. Acta Pharmacologica Sinica 2011;32:716-720.

[2] Richard C, Gao J, Brown N, Reese J. Aquaporin water channel genes are differentially expressed and regulated by ovarian steroids during the periimplantation period in the mouse. Endocrinology 2003;144:1533-1541.

[3] Cullinan-Bove K, Koos RD. Vascular endotelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. Endocrinology 1993;133:829–837.

[4] Damiano AE. Review: Water channel proteins in the human placenta and fetal membranes. Placenta 2011;25:S207-S211.

[5] Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, Bazer FW, Spencer TE. Developmental biology of uterine glands. Biol Reprod 2001;65:1311–1323.

[6] Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yo- shinaga K. Embryo implantation. Dev Biol 2000;223:217–237.

[7] Li X, Yu HM, Koide SS. The water channel gene in human uterus. Biochem Exp Biol 1994;32:371-377.

[8] Gannon BJ, Warnes GM, Carati CJ, Verco CJ. Aquaporin-1 expression in visceral smooth muscle cells of female rat reproductive tract. J Smooth Muscle Res 2000;36:155-167.

[9] Lindsay LA, Murphy CR. Redistribution of aquaporins 1 and 5 in the rat uterus is dependent on progesterone: a study with light and electron microscopy. Reproduction 2006;131:369-378.

[10] Skowronski MT, Kwon TH, Nielsen S. Immunolocalization of aquaporin 1, 5, and 9 in the female pig reproductive system. J Histochem Cytochem 2009;57:61-67.

[11] Aralla M, Borromeo V, Groppetti D, Secchi C, Cremonesi F, Arrighi S. A collaboration of aquaporins handles water transport in relation to the estrous cycle in the bitch uterus. Theriogenology 2009;72:310-321.

[12] Echevarria M, Windhager EE, Tate SS, Frindt G. Cloning and expression of AQP3, a water channel from the medullary collecting duct of rat kidney. Prod Natl Acad Sci 1994;91:10997-1001.

[13] Umenishi F, Verkman A, Gropper M. Quantitiative analysis of aquaporin mRNA expression in rat tissues by RNase protection assay. DNA Cell Biol 1996;15, 475–480.

[14] Mobasheri A, Wray S, Marples D. Distribution of AQP2 and AQP3 water channels in human tissue microarrays. J Mol Hist 2005;36:1-14.

[15] Jablonski EM, McConell NA, Hughes FM Jr, Huet-Hudson YM. Estrogen regulation of aquaporins in the mouse uterus: potential roles in uterine water movement. Biol Reprod 2003;69, 1481-1487.

[16] Johnston T and Wintour EM. Ontogeny of aquaporins 1 and 3 in ovine placenta and fetal membranes. *Placenta* 2000;21, 88–99.

[17] Tani T, Koyama Y, Nihei K, Hatakeyama S, Ohshiro K, Yoshida Y, Yaoita E, Sakai Y, Hatakeyama K, Yamamoto T. Immunolocalization of aquaporin-8 in rat digestive organs and testis. Arch Histol Cytol 2001;64:159-68.

[18] Gao Y, Deng Q, Zhang Y, Zhang S, Zhu Y, Zhang J. The expression of the multiple splice variants of AQP8 in porcine testes at different developmental stages. J Appl Genet 2014;55:511-4.

[19] Ma T, Yang B, Verkman AS. Cloning of a novel water and urea-permeable aquaporin from mouse expressed strongly in colon, placenta, liver, and heart. Biochem Biophys Res Commun 1997;240:324-8.

[20] Wang S, Kallichanda N, Song W, Ramirez BA, Ross MG. Expression of aquaporin-8 in human placenta and chorioamniotic membranes: evidence of molecular mechanism for intramembranous amniotic fluid resorption. *Am J Obstet Gynecol* 2001;185, 1226–31.

[21] Qi HB, Li L, Zong WJ, Hyer BJ, Huang J. Expression of aquaporin 8 is diversely regulated by osmotic stress in amnion epithelial cells. *J Obstet Gynaecol* 2009;35, 1019-25.

[22] Knospe C. Periods and stages of the prenatal development in the domestic cat. Anat. Histol. Embryol 2002;**31**, 37-51.

[23] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976;72, 248–256.

[24] Sirois J and Doré M. The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process. Endocrinology 1997;138:4427–4434.

[25] Clemetson C, Verma U, De Carlo S. Secretion and reabsorption of uterine luminal fluid in rats. J Reprod Fertil 1977;49:183-187.

[26] Chaves M, Ribeiro R, Rao V. Possible involvement of nitric oxide in estrogen-induced uterine edema in the immature rat. Braz J Med Biol Res 1993;26:853-857.

[27] Richard C, Gao J, Brown N, Reese J. Aquaporin water channel genes are differentially expressed and regulated by ovarian steroids during the periimplantation period in the mouse. Endocrinology 2003;144:1533-1541.

[28] Aralla M, Mobasheri A, Groppetti D, Cremonesti F, Arrighi S. Expression of aquaporin water channels in canine fetal adnexa in respect to the amniotic fluid production and absoption. Placenta 2012;22:502-510.

[29] Skowronska A, Mlotkowska P, Nielsen S, Skowronski MT. Difference in expression between AQP1 and AQP5 in porcine endometrium and myometrium in response to steroid hormones, oxytocin, arachidonic acid, forskolin and cAMP during the mid-luteal phase of the estrous cycle and luteolysis. Reprod Biol Endocrinol 2015;1; 13:131.

436 [30] Li X, Yu H, Koide SS. Regulation of water channel gene (AQP- CHIP) expression
437 by estradiol and anordiol in rat uterus. Yao Xue Xue Bao 1997;32:586–92.
438 [31] Zu X.Q, Jiang XS, Zhu XJ, Zou SW, Wang YH, Hu YC. Expression of Aquaporin
439 1 and Aquaporin 3 in fetal membranas and placenta in human term pregnancies with
440 olygohidramnios. Placenta 2009;30: 670-676.
441 [32] Mann SE, Ricke EA, Yang BA, Verkman AS, Taylor RN. Expression and
442 localization of aquaporin 1 and 3 in human fetal membranes. Am J Obstet Gynecol
443 2002;187(4):902e7.
444 [33] Beall MH, Wang S, Yang B, Chaudhri N, Amidi F, Ross MG. Placental and
445 membrane aquaporin water channels: Correlation with amniotic fluid volumen and
446 composition. 2007;28:421-8.
447 [34] Wakayama Y, Jimi T, Inoue M, Kojima H. Shibuya S, Murahashi M, Hara H,
448 Oniki H. Expression of aquaporin 3 and its localization in normal skeletal myofibres.
449 Histochem J 2002;34: 331– 337.
450 [35] Damiano A, Zotta E, Goldstein J, Reisin I, Ibarra C. Water channel proteins AQP3
451 and AQP9 are present in syncitiotrophoblast of human term placenta. Placenta 2001;22:
452 776-781.
453 [36] Johnston H, Koukoulas I, Jeyaseelan K, Armugam A, Earnest L, Baird R, Dawson
454 N, Ferraro T, Wintour EM. Ontogeny of aquaporins 1 and 3 in ovine placenta and fetal
455 membranas. Placenta 2000;21: 88-99.
456 [37] Brosens IA, Robertson WB, Dixon HG. The role of the spiral arteries in the
457 pathogenesis of preeclampsia.Obstet Gynecol Ann. 1972;1:177–191.
458 [38] Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the
459 development puzzle. Science. 1994;266:1508–1518.
460 [39] Crocker IP, Strachan BK, Lash GE, Cooper S, Warren AY, Baker PN. Vascular
461 endothelial growth factor but not placental growth factor promotes trophoblast
462 syncytialization in vitro. J Soc Gynecol Investig. 2001;8:341–346.
463 [40] Nelson DM, Crouch EC, Curran EM, Farmer DR. Trophoblast interaction with
464 fibrin matrix. Am J Pathol. 1990;136:855–865.
465 [41] Torry DS, Ahn H, Barnes EL, Torry RJ. Placenta growth factor: potential role in
466 pregnancy. Am J Reprod Immunol. 1999;41:79–85.
467 [42] Mathieu CL, Burnett SH, Mills SE, Overpeckt JG, Bruns DE, Bruns ME.
468 Gestational changes in calbindin-D9k in rat uterus, yolk sac, and placenta: implications
469 USA 1989;86:3433-3437.

[43] Lindsay LA and Murphy CR. Redistribution of aquaporins 1 and 5 in the rat uterus is dependent on progesterone: a study with Light and electron microscopy. Reproduction 2006;131:369-378.

[44] Liu H, Zheng Z, Wintour EM. Aquaporins and fetal fluid balance. Placenta 2008;29:840e7.

[45] Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. J Perinatol 2005;25:341e8.

Figure legends

Fig 1. Western blotting expression for A) AQP1, B) AQP3, and C) AQP8. Radiograph films show a 28-32 kDa band compatible with the different AQPs. C+: Positive control; C-: Negative control; MK: molecular weight marker; LP: non-pregnant queens with low levels of serum progesterone; HP: non-pregnant queens with high levels of serum progesterone; UT: uterus; TZ: transference zone; D30: 30 days of pregnancy; D40: 40 days of pregnancy; D50: 50 days of pregnancy; D60: 60 days of pregnancy. No statistically significant difference among the different groups was observed.

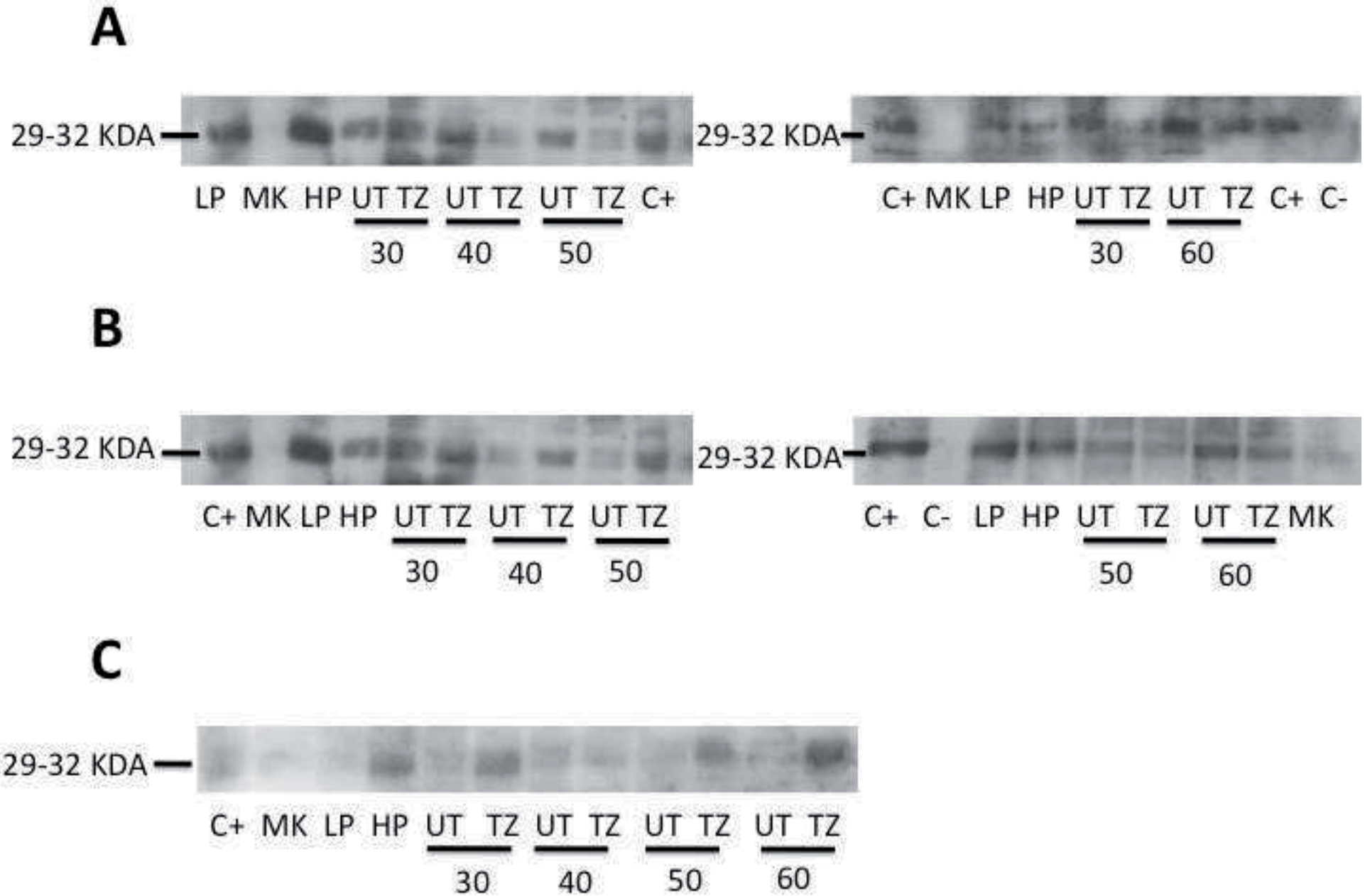
Fig 2. Immunohistochemistry images for AQP-1 expression. Cellular nuclei are blue-stained with DAPI dye, while AQP-1 is red-stained. **A:** Negative control in a uterine sample; **B:** Positive control in a rat kidney sample. Note the red mark for AQP-2 around the collecting ducts membrane of the kidney; **C:** Uterine sample from a non-pregnant queen with low levels of serum progesterone. Note the red mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **D:** Uterine sample from a non-pregnant queen with high levels of serum progesterone. Note the red mark in the cytoplasm of the cells from the glandular and luminal epithelia (white arrows) and the red mark in the myometrium (white asterisk); **E:** Uterine sample from D30 pregnancy. Note the red mark in the cytoplasm of the cells from the luminal and glandular epithelia (white arrow); **F:** Uterine sample from D40 pregnancy. Note the red mark in the cells from the endometrial glands and luminal epithelia (white arrow); **G:** Uterine sample from a D50 pregnancy. Note the red mark in the cytoplasm of the cells from the luminal epithelium (white arrow); **H:** Uterine sample from a D60 pregnancy. Note the red mark in the cytoplasm of the cells from the

503 luminal epithelium (white arrow) and the red mark in the vascular endothelia (white
504 asterisk); **I:** Sample from the placental transference zone in a D30 pregnancy. No
505 specific staining was noticed in the chorionic membranes; **J:** Sample from the placental
506 transference zone in a D40 pregnancy. No specific staining was noticed in the chorionic
507 membranes; **K:** Sample from the placental transference zone in a D50 pregnancy. No
508 specific staining was noticed in the chorionic membranes; **L:** Sample from the placental
509 transference zone in a D60 pregnancy. No specific staining was noticed in the chorionic
510 membranes.
511
512 Fig 3. Immunohistochemistry images for AQP-3 expression. Cellular nuclei are blue-
513 stained with DAPI dye, while AQP-3 is green-stained. **A:** Negative control in a uterine
514 sample; **B:** Positive control in a rat kidney sample. Note the green mark for AQP-3
515 around the collecting ducts membrane of the kidney; **C:** Uterine sample from a non-
516 pregnant queen with low levels of serum progesterone. Note the green mark in the
517 cellular membrane of the epithelial cells from the glandular and luminal epithelia (white
518 arrows) and the green mark in the myometrium (white asterisk); **D:** Uterine sample
519 from a non-pregnant queen with high levels of serum progesterone. Note the green mark
520 in the cytoplasm of the cells from the glandular and luminal epithelia (white arrows)
521 and the green mark in the myometrium (white asterisk); **E:** Uterine sample from D30
522 pregnancy. Note the green mark in the cytoplasm of the cells from the luminal and
523 glandular epithelia (white arrow); **F:** Uterine sample from D40 pregnancy. Note the
524 green mark in the cells from the endometrial glands and luminal epithelia (white arrow);
525 **G:** Uterine sample from a D50 pregnancy. Note the green mark in the cytoplasm of the
526 cells from the luminal epithelium (white arrow); **H:** Uterine sample from a D60
527 pregnancy. Note the green mark in the cytoplasm of the cells from the luminal

528 epithelium (white arrow); **I:** Sample from the placental transference zone in a D30
529 pregnancy. Note the green mark in the cytoplasm of the cells from the labyrinth (white
530 asterisk); **J:** Sample form the placental transference zone in a D40 pregnancy. Note the
531 green mark in the cytoplasm of the cells from the labyrinth (white asterisk); and the
532 specific green mark in the cytotrophoblasts (white arrow); **K:** Sample from the placental
533 transference zone in a D50 pregnancy. Note the green mark in the cytoplasm of the cells
534 from the labyrinth (white asterisk); and the specific green mark in the cytotrophoblasts
535 (white arrow); **L:** Sample from the placental transference zone in a D60 pregnancy.
536 Note the green mark in the cytotrophoblasts (white arrow).
537
538 Fig 4. Immunohistochemistry images for AQP-8 expression. Cellular nuclei are blue-
539 stained with DAPI dye, while AQP-8 is purple-stained. **A:** Negative control in a rat
540 kidney sample; **B:** Positive control in a rat kidney sample. Note the purple mark for
541 AQP-8 around the collecting ducts membrane of the kidney; **C:** Uterine sample from a
542 non-pregnant queen with low levels of serum progesterone. Note the purple mark in the
543 cellular membrane of the epithelial cells from the glandular and luminal epithelia (white
544 arrows); **D:** Uterine sample from a non-pregnant queen with high levels of serum
545 progesterone. Note the purple mark in the cytoplasm of the cells from the glandular and
546 luminal epithelia (white arrows) and the purple mark in the myometrium (white
547 asterisk); **E:** Uterine sample from D30 pregnancy. Note the purple mark in the
548 cytoplasm of the myocytes from the myometrium (white asterisk) and the cells from the
549 endometrial glands (white arrow); **F:** Uterine sample from D40 pregnancy. Note the
550 purple mark in the cytoplasm of the myocytes from the myometrium (white asterisk)
551 and the cells from the endometrial glands (white arrow); **G:** Uterine sample from a D50
552 pregnancy. Note the purple mark in the cytoplasm of the cells from the luminal

553 epithelium (white arrow) and glandular epithelium (white asterisk); **H:** Uterine sample
554 from a D60 pregnancy. Note the purple mark in the cytoplasm of the cells from the
555 luminal epithelium (white arrow) and glandular epithelium (white asterisk); **I:** Sample
556 from the placental transference zone in a D30 pregnancy. Note the purple mark in the
557 cytoplasm of the cells from the labyrinth (white arrow); **J:** Sample form the placental
558 transference zone in a D40 pregnancy. Note the purple mark in the cytoplasm of the
559 cells from the labyrinth (white arrow); **K:** Sample from the placental transference zone
560 in a D50 pregnancy. Note the purple mark in the cytoplasm of the cells from the
561 labyrinth (white arrow); Note the purple mark in the cytoplasm of the cells from the
562 labyrinth (white arrow); **L:** Sample from the placental transference zone in a D60
563 pregnancy. Note the purple mark in the cytoplasm of the cells from the labyrinth (white
564 arrow).
565
566 Fig 5. Immunohistochemistry image from a non-pregnant queen with low levels of
567 serum progesterone (LP). Cellular nuclei are blue-stained with DAPI dye. AQP-8
568 (purple mark) is distributed only in the cellular membrane of the cells from the
569 glandular and luminal epithelium (white arrow).
570
571 Table 1. Relative abundance of AQP1, -3 and -8 calculated by means of the Multi
572 Gauge v3.0 software system. Statistically significant differences at different sexual and
573 pregnancy stages for each AQP are indicated with different superscripts.
574
575 Table 2. Correlations between AQPs expression and serum levels of progesterone.
576 Statistically significant differences are indicated with asterisks.
577

Figure
Click here to download high resolution image



Figure

Click here to download high resolution image

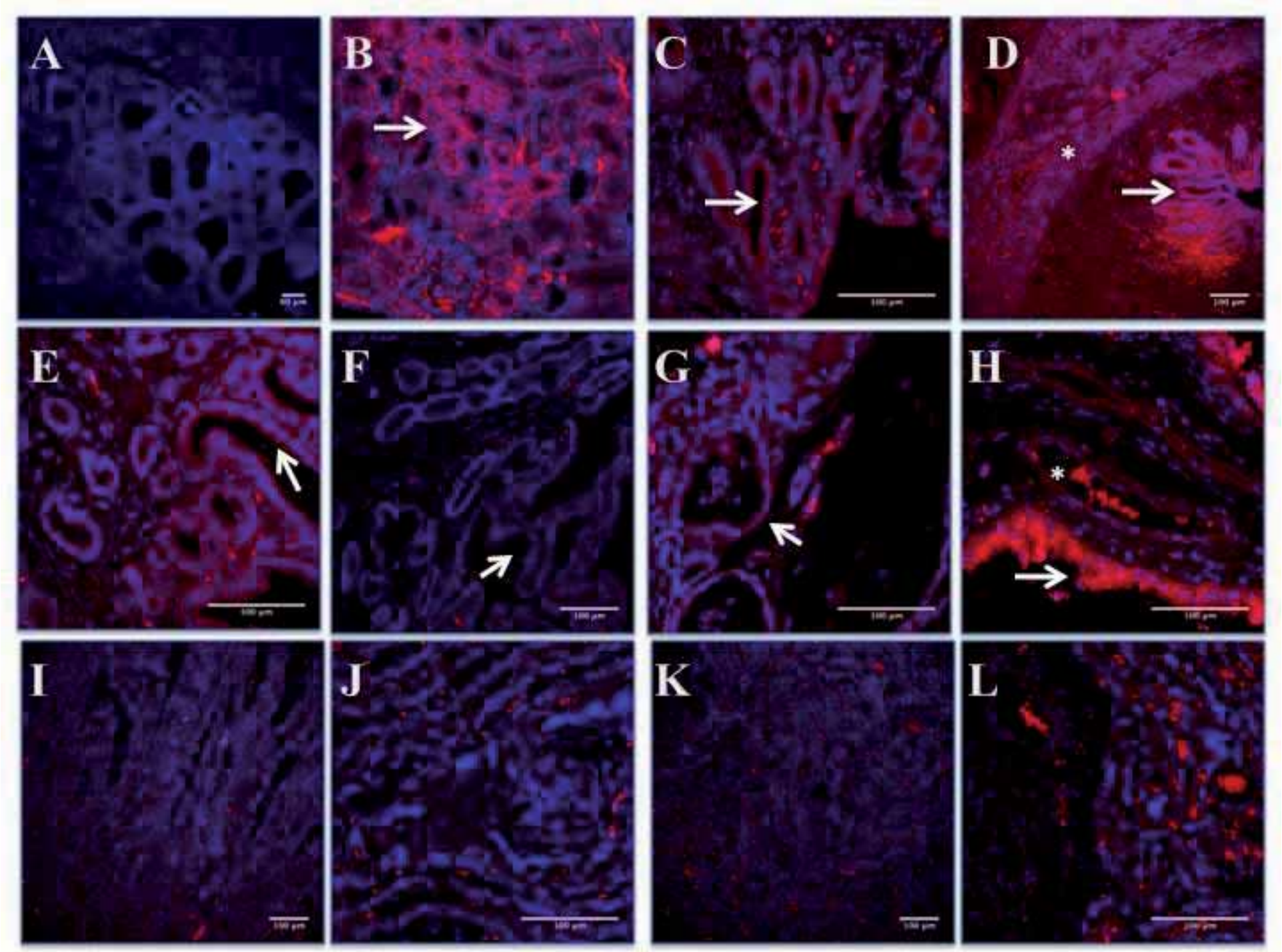
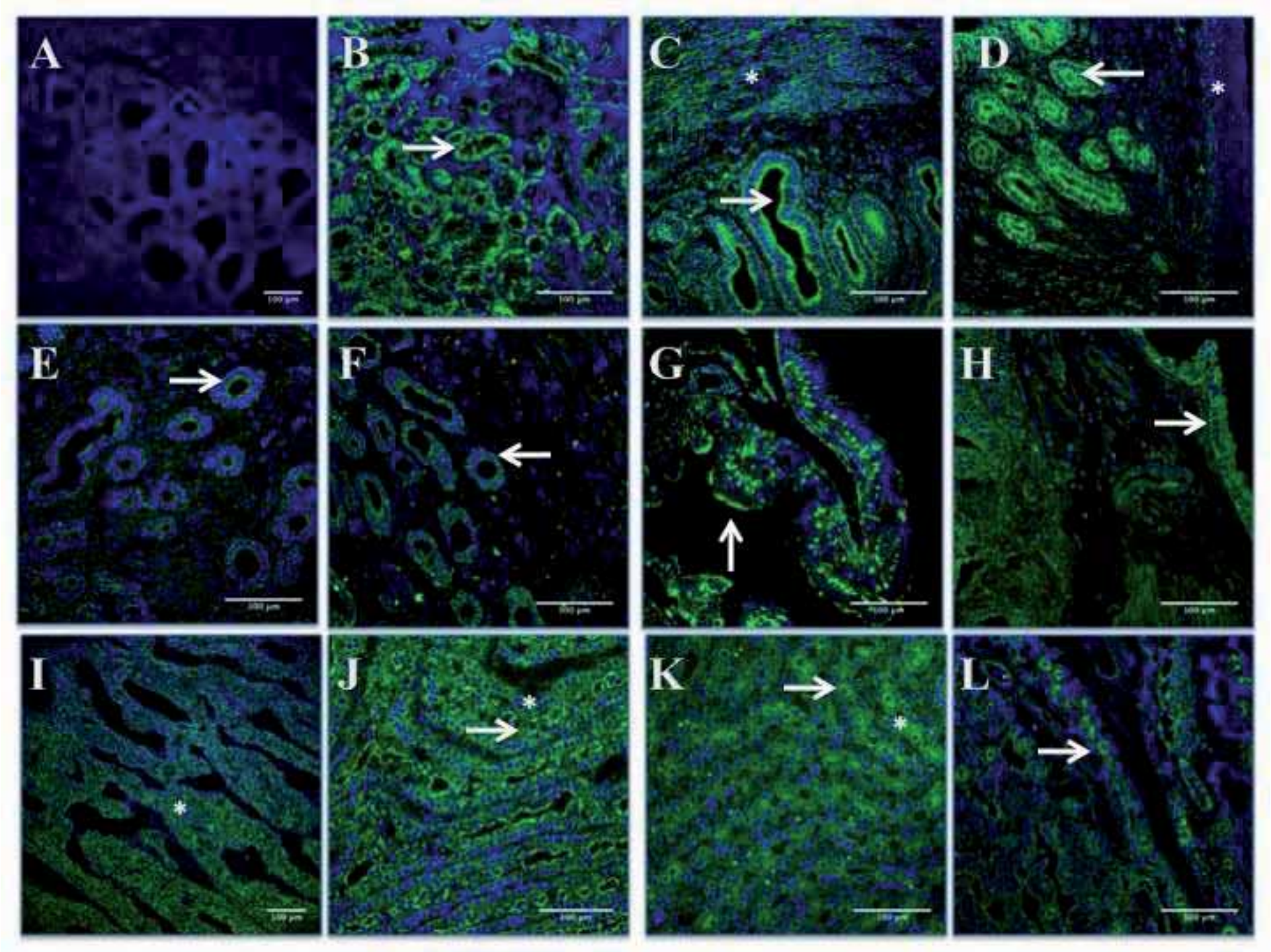
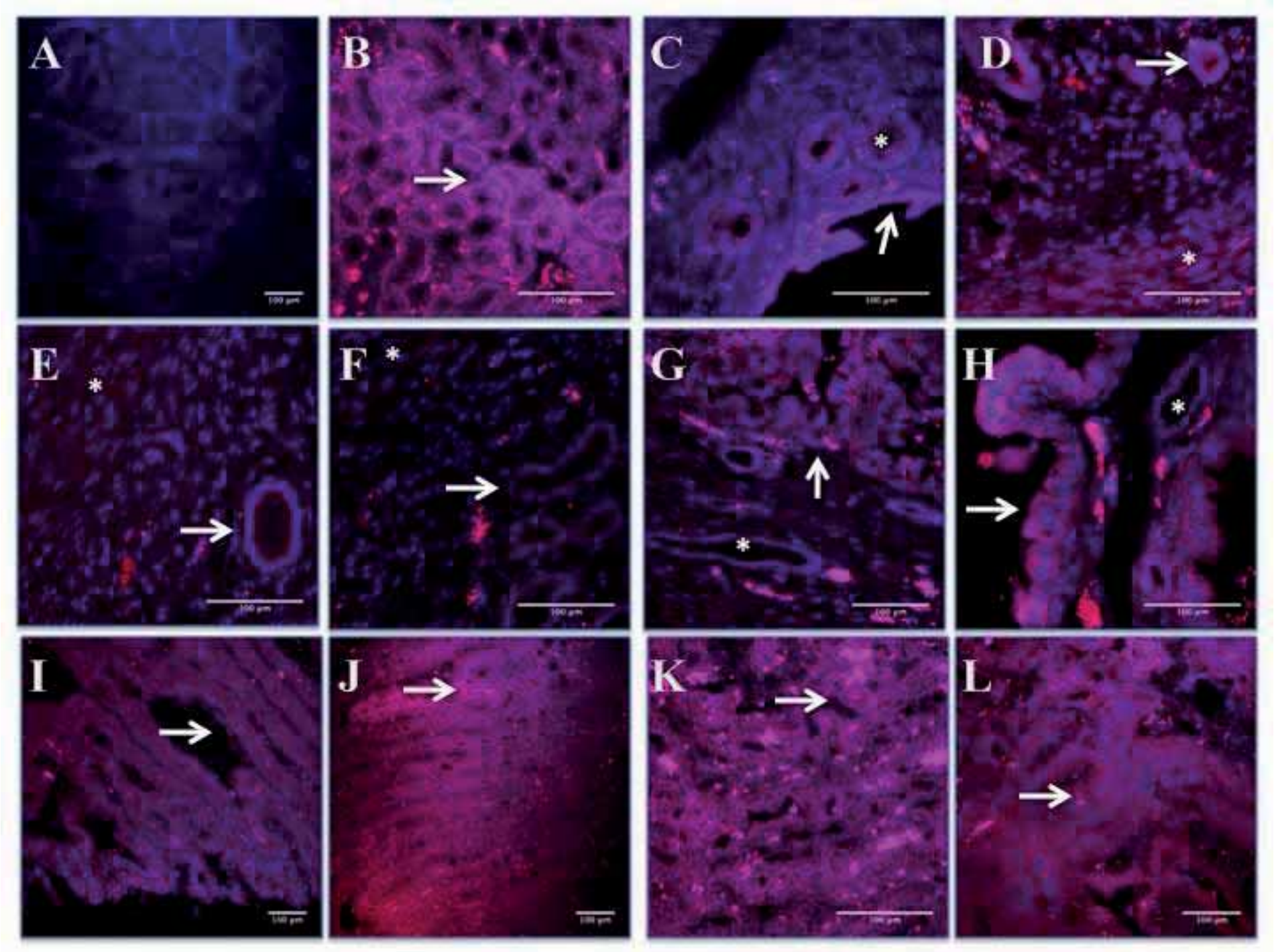


Figure
Click here to download high resolution image



Figure

Click here to download high resolution image



Figure

Click here to download high resolution image

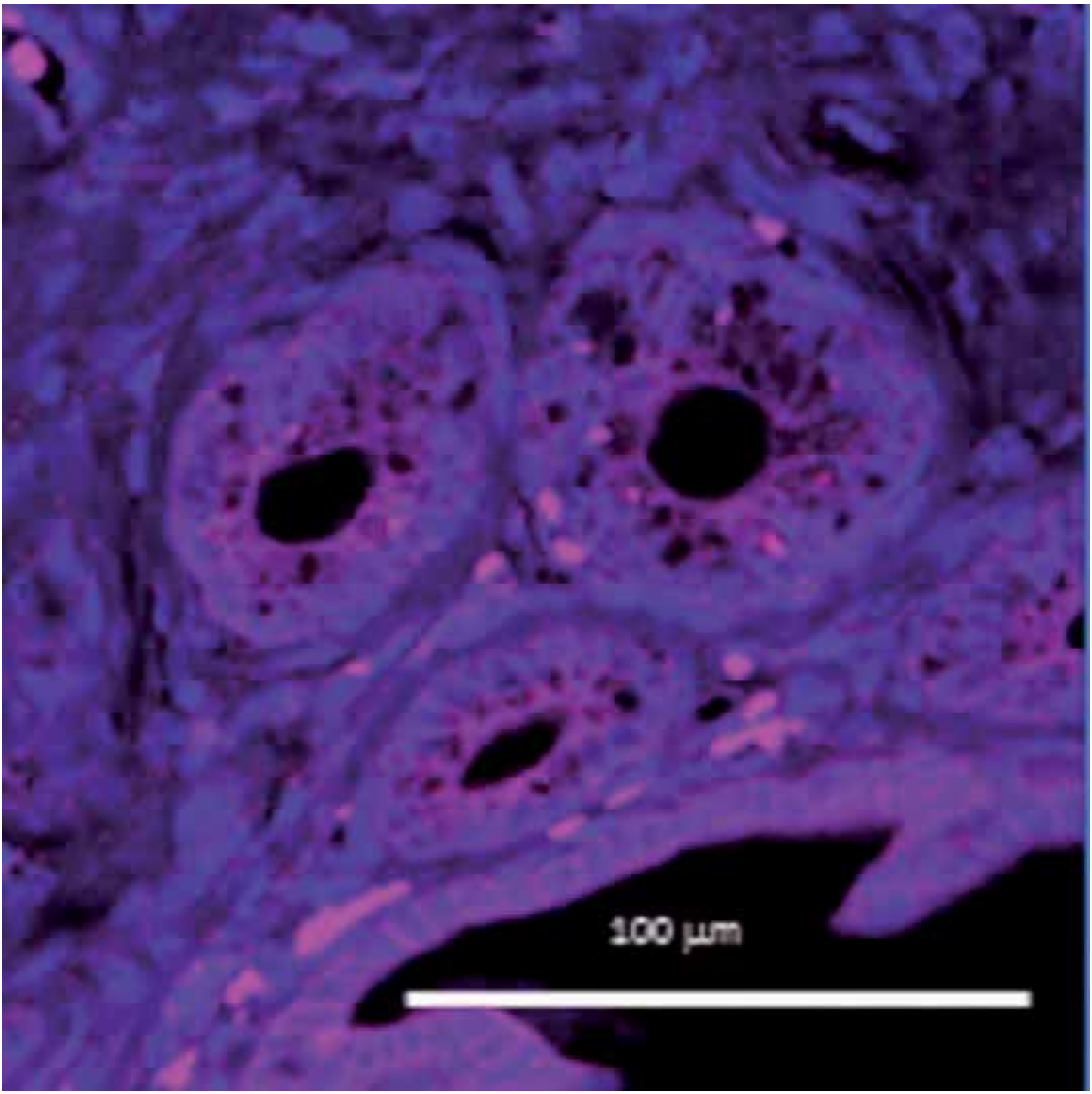


Table 1

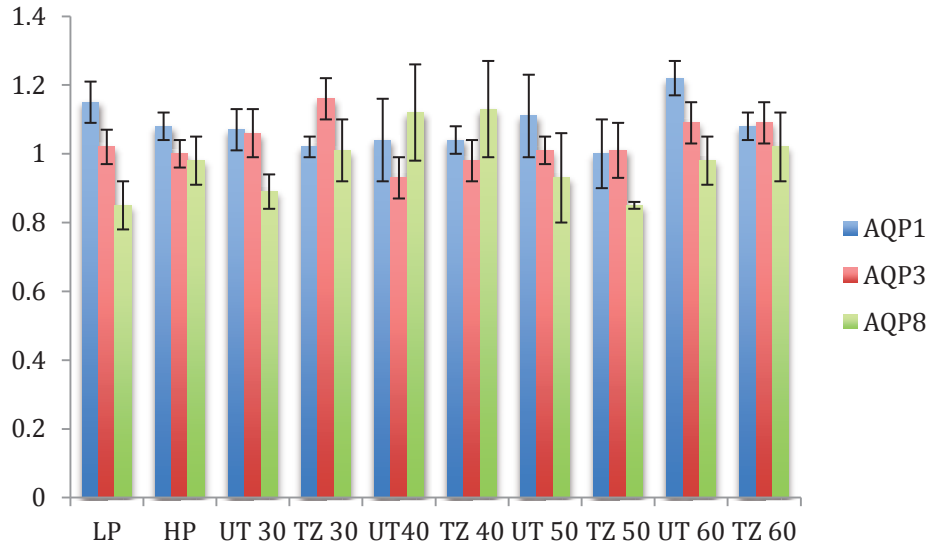


Table 2

	AQP1		AQP3		AQP8		P4	
AQP1		1	0.35*		0.16		-0,32*	
AQP3	0.35*			1	0.29			-0,19
AQP8	0.16		0.29			1	0.18	
P4	-0,32*			-0,19	0.18			1

Highlights

- Feline endometrium and placenta express aquaporin-1, -3 and -8 (AQP1, -3, -8)
- Gestational age and progesterone levels do not induce changes in AQP1, -3 and -8 expression
- Levels of serum progesterone induce changes in the cellular location of AQP-8

Dear Sir/Madam,

Enclosed please find our manuscript entitles **Aquaporin-1, -3 and -8 in queen reproductive tract may handle water transport on queen reproductive tract and placental transfer zone. Evaluation at different phases of the sexual cycle and pregnancy stages**. The manuscript pretends to stablish the putative role of aquaporins 1, 3 and 8 in the reproductive function, especially during foetal development in queens. The role of aquaporins has been previously described in other mammal species, but this is the first study focused in cats. The expression of aquaporin has been described in the manuscript and also its relationship with progesterone levels.

I hope you consider the present manuscript for publication in your journal.

Sincerely,

MM Rivera del Alamo

DVM, PhD, ECAR Diplomate

Click here to download high resolution image

Theriogenology
Author Agreement Form

This Form should be signed by all authors OR by the corresponding (or senior) author who can vouch for all co-authors. A scanned copy of the completed Form may be submitted online.

The authors confirm the following statements:

1. that there has been no duplicate publication or submission elsewhere of this work
2. that all authors have read and approved the manuscript, are aware of the submission for publication and agree to be listed as co-authors

Author Name	Signature
LLUÍS PERRÉ-DOLCET	
MARC YESTE	
MERITXELL VENDRELL	
TERESA RIGAU	
JOAN ENRIC RODRIGUEZ-GIL	
M. MONTSERRAT RIVERA DEL ALAMO	

Article 2: Aquaporin-2 expression in the uterus and placental transference zone at different gestacional and non-gestational phases in the queen.

Reproduction, Fertility and Development

Aquaporin 2 expression in the uterus and placental transference zone at different gestational and non-gestational phases in the queen

Journal:	*Reproduction, Fertility and Development*
Manuscript ID	Draft
Manuscript Type:	Research paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Ferre-Dolcet, Lluis; Autonomous University of Barcelona, Department of Animal Medicine and Surgery Yeste, Marc; University of Girona, Cell Biology; University of Oxford, Nuffield Department of Obstetrics and Gynaecology Vendrell, Meritxell; Autonomous University of Barcelona, Department of Animal Medicine and Surgery Rodriguez-Gil, Joan; Autonomous University of Barcelona, Dept. Animal Medicine and Surgery Rigau, Teresa; Autonomous University of Barcelona, Dept. Animal Medicine and Surgery Rivera del Alamo, Maria Montserrat; Autonomous University of Barcelona, Department of Animal Medicine and Surgery
Keyword:	endometrium, placenta, pregnancy, progesterone

Title

Aquaporin 2 expression in the uterus and placental transference zone at different gestational and non-gestational phases in the queen

Running title

AQP2 in cat female reproductive tract

Authors

Lluís Ferré-Dolcet[1], Marc Yeste[2], Meritxell Vendrell[1], Joan Enric Rodríguez-Gil[1], Teresa Rigau[1*] Maria Montserrat Rivera del Alamo[1*]

Affiliation

[1]Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, E-08193 Bellaterra (Cerdanyola del Vallès), Spain

[2]Department of Biology, University of Girona, Girona, E-17071 Girona, Spain

***Corresponding authors**

Departament de Medicina i Cirurgia Animals, Facultat de Veterinària, Edifici V, Campus Universitari, Universitat Autònoma de Barcelona, 08193 Bellaterra (Cerdanyola del Vallès), Spain. Tel.: +34 935811045. E-mail addresses: mariamontserrat.rivera@uab.cat; teresa.rigau@uab.cat

Abstract

Aquaporins are present in many tissues, including reproductive organs. The present study was focused on the localization of aquaporin-2 (AQP2) during separate gestational and non-gestational phases in the cat. Queens were distributed into six groups: non-macroscopically pregnant with high levels of progesterone, non-macroscopically pregnant with low levels of progesterone, and at 30, 40, 50 and 60 days of pregnancy. Immunoblotting identified a 29-kDa band compatible with AQP2, but no significant differences in the relative levels were observed between groups. Immunohistochemistry showed that AQP2 was located in the myometrium, luminal and glandular epithelia, and chorionic labyrinth cells. While AQP2 was located in the membrane of luminal and glandular epithelia cells in queens with low levels of progesterone, it was evenly distributed all over the cell in queens with high levels of progesterone regardless the gestational age. Moreover, no significant changes in the tyrosine phosphorylation levels of AQP2 were observed in any experimental group. In conclusion, AQP2 has been described for the first time in the reproductive tract of pregnant and non-pregnant queens. Furthermore, our results suggest that AQP2 plays a role in the regulation of water metabolism during queen pregnancy that would be related with gestational-linked changes of serum progesterone levels.

Keywords: Aquaporin-2, pregnancy, queen, endometrium, transference zone

Introduction

As water is considered to be the main molecular form for life development, its role on fetus development is apparent. Indeed, total fetal water requirements increase markedly due to an exponential growth in fetal weight over pregnancy (Woo *et al.* 1985). The placenta is the major organ that regulates the exchange of solutes and nutrients between the conceptus and the dam. Because of this function, both uterus and placenta concomitantly undergo several structural changes during pregnancy to prepare the endometrium for implantation and further fetal development. The glandular epithelium provides histiotrophic nutrition to the fetus, mostly during mid-pregnancy (Miglino *et al.* 2006). Furthermore, and in order to maintain the fetus healthy and nourished, fetal membranes start to progress, encroaching to the maternal layers and increasing its thickness at late pregnancy (Miglino *et al.* 2006).

The queen placenta is microscopically defined as endotheliochorial, which means that chorion erodes the endometrial epithelium and directly contacts the maternal vascularization, creating an intermingled system. Fluid movements across cell membranes of the uterus are important for both implantation and pregnancy (Miglino *et al.* 2006). This water movement across a variety of biological membranes is regulated by aquaporins (AQPs), which are small, hydrophobic membrane proteins that form water channels (Agre *et al.* 2002*;* Richard *et al.* 2003*)*. Thirteen AQPs have been described in mammals and are divided into three groups. The first group is that of actual AQPs, which are water-selective channels. AQP1, AQP2, AQP4 and AQP5 are members of this subfamily. The second group known as aquaglyceroporins, are proteins (AQP3, AQP7, AQP9 and AQP10) that not only are able to transport water but also other solutes such as glycerol. Finally, unorthodox aquaporins (or superaquaporins), the

third group that comprises AQP6, AQP8, AQP11 and AQP12, which function remains largely unclear (Park and Kwon, 2015).

AQP2 is a 29-kDa protein that was originally identified in kidney collecting ducts (Fushimi *et al.* 1993). In kidney, AQP2 acts as a vasopressin-regulated water channel of the distal nephrons, thus regulating the volume and osmolarity of urine (Sasaki *et al.* 2000). Lately, several authors described the presence of AQP2 in extra-renal localizations, including male and female reproductive tracts (Stevens *et al.* 2000*;* Hildenbrand *et al.* 2006*)*. Regarding female genital tract, AQP2 has been found in the luminal and glandular epithelium of mouse (Jablonski *et al. 2003)* and human endometrium (Nejsum *et al.* 2005*;* He *et al.* 2006*)* and its relative abundance in the endometrium has been correlated with serum levels of estradiol (17β-E_2) and progesterone (P_4) (Jablonski *et al.* 2003*;* He *et al.* 2006*)*.

Taking into account all of the aforementioned, the main aim of the current work was to establish the presence of AQP2 in the uterus and the placental transference zone of queens at different gestational and non-gestational phases. This main aim was tackled through three specific points. The first point consisted of determining the presence of AQP2 in the queen endometrium during different gestational ages in order to evaluate changes in the amount and/or localization during pregnancy. The second point was to examine the putative relationship between uterine and placental AQP2 levels and serum progesterone (P_4) concentration. The third point was to determine whether variations in AQP2 activity during queen pregnancy could be mediated by changes in the tyrosine phosphorylation levels of AQP2.

Materials and methods

Animals and sample collection

Forty-two queens were included in the present study. The females belonged to a stray-cats sterilization program performed at the Universitat Autònoma de Barcelona (Spain). Inclusion criteria were no sign of current illness on physical examination and negative to feline leukemia (FeLV) and feline immunodeficiency (FIV) virus. Samples were collected according to the guidelines of the Ethical Committee Animal Care and Research, Autonomous University of Barcelona (Bellaterra, Spain. CEEAH code 2939). Prior to surgery, queens were pre-medicated (i.m.) with ketamine (5 mg/kg, Imalgene 1000, Laboratorios Merial, Barcelona, Spain), buprehorphine (20 μg/kg, Buprecare, Divasa-Farmavic S.A, Gurb, Spain) and midazolam (0.2 mg/kg, Midazolam Normon, Laboratorios Normon S.A., Madrid, Spain). Once pre-medication was effective, a blood sample from the jugular vein was obtained. Females were then induced with i.v. propofol (4 mg/kg, Vetofol, Laboratorios Esteve, Barcelona, Spain) and the trachea was intubated. Anesthesia was maintained with 1.5-2% isoflorane (Isoflo, Laboratorios Esteve, Barcelona, Spain) in oxygen through a Mapleson F anesthetic circuit. Following this, queens underwent standard ovariohysterectomy by midline laparotomy. Once the uterus was removed, pregnancy was confirmed or discarded by macroscopic observation. When non-pregnant, queens were divided into ovulated (n = 8) and non-ovulated (n = 8) according to serum concentration of progesterone. When pregnant, queens were divided according to their gestational age into four groups, 30 (D_{30}; n = 8), 40 (D_{40}; n = 5), 50 (D_{50}; n = 4) and 60 (D_{60}; n = 9) days of pregnancy, following Knospe (Knospe 2002) and Fresno et al. (Fresno *et al. 2012)*.

From both non-pregnant and pregnant queens, a complete sectional sample of uterine horn was collected and split into two fractions. One was fixed with 10% (w:v) paraformaldehyde. The other fraction was resected to obtain the uterine mucosa, which was snap frozen in liquid nitrogen and kept at -80ºC until analysis. In addition, two samples from the transference zone of the placenta were also obtained from pregnant queens and processed through the same way. One was fixed in 10% (w:v) paraformaldehyde, whereas the other was snap frozen in liquid nitrogen and kept at -80ºC until analyzed. Samples fixed with 10% (w:v) paraformaldehyde were used for immunohistochemistry purposes, while frozen samples were used for immunoblotting and immunoprecipitation.

Progesterone concentration

Blood samples were collected into glass tubes and allowed to clot for 10 minutes. Samples were then centrifuged for 7 minutes at 2500*g* and stored at -20ºC until analysis. Serum progesterone concentration was determined with Immulite® 1000 equipment (Immulite®, Siemens Healthcare Diagnostics, Cornellà del Llobregat, Spain).

Progesterone levels were used to determine ovulation in non-pregnant queens following the criteria set by Fresno et al. (Fresno *et al.* 2012). Thus, queens with progesterone concentration ≤1 ng/mL were included in the non-ovulated group (low progesterone, LP), whereas those with progesterone concentration ≥2 ng/mL were included in the ovulated group (high progesterone, HP). Queens with values between 1 and 2 were not included in the study.

Immunoblotting

Frozen tissue samples were homogenized in 1 mL of cold protein extraction buffer (50 mM Tris(hydroxymethyl)aminomethane hydrochloride (TRIS HCl) pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethylene glycol tetraacetic acid (EGTA), 25 mM dithiothreitol (DTT), 1.5% (w:v) Triton-X-100, 1 mM phenylmethane sulfonyl fluoride (PMSF), 10 µg/mL leupeptine, 1 mM Na_2VO_4, 1 mM benzamidine) using an Ultra-Turmax T25 basic homogenizer (IKA-WERKE, Staufen, Germany). Samples were then centrifuged at 4ºC at 2500*g* for 5 minutes and supernatants were recovered.

Protein concentration of supernatants was determined through the Bradford technique (Bradford 1976) utilizing a commercial kit (BioRad, Hercules, CA, USA). Afterwards, proteins were separated by SDS-PAGE electrophoresis in 10% (w:v) acrylamide gels and transferred to nitrocellulose membranes according to Sirois and Dore (Sirois and Doré 1997). Detection of AQP2 was performed with a rabbit anti-AQP2 antibody (Novus Biologicals, Abingdon, United Kingdom) at a dilution of 1:1,000 (v:v). A donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., 10410 Finnell Street, Dallas, TX, USA) was used as secondary antibody at a dilution of 1:1,000. Membranes were exposed to radiograph films to reveal AQPs bands (28-32 kDa) after 5 minutes incubation with Immobilon TM Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA). A sample of rat kidney was used as positive control.

After detection of AQP2, membranes were exposed to a stripping buffer (1% (w:v) glycine, 0.1% (w:v) sodium dodecyl sulfate, 1% (w:v) Tween 20, pH 2.2) to remove the specific AQP2 marking and subsequently re-probe with a specific anti-mouse α-tubulin antibody (ABR Affinity BioReagents, CO, USA). This was used as an

internal control to verify that the same amount of protein from each sample was loaded in all lanes. Finally, a computer-assisted image analysis system (Multi Gauge v 3.0 software system, Fujifilm, Tokyo, Japan) was utilized to assess the integral densities of the obtained AQP2 and α-tubulin specific bands, and to standardize AQP2 against α-tubulin, as relative AQP2-amounts.

Immunohistochemistry

Fixed samples were kept in 10% (w:v) paraformaldehyde for 48 hours at 20ºC and then embedded in paraffin blocks. Blocks were cut with a microtome and 5 mm-thick sections were obtained. In order for the sections to be de-waxed, they were subsequently immersed twice in xylene, twice in 100% (v:v) ethanol, twice in 96% (v:v) ethanol and, finally twice in 70% (v:v) ethanol. Each immersion step lasted 5 minutes. Following de-waxing, sections were washed twice in phosphate buffered saline (PBS; pH 7.4) and then immersed for 20 minutes in citrate buffer (pH 6.0) at 96ºC for epitope unmasking. This immersion was followed by a washing step for 10 minutes in PBS at room temperature.

After washing, sections were incubated in an aqueous 2% (w:v) bovine serum albumin (BSA) solution for one hour at 20ºC. Afterwards, sections were incubated with the above described specific anti-AQP2 antibody at a dilution of 1:100 (v:v) for 8 hours at 4ºC. Sections were then washed for 5 minutes in PBS and further incubated for 3 hours at 4ºC with a secondary Alexa Fluor 647-conjugated, anti-rabbit antibody at a dilution of 1:100 (v:v). Then, sections were washed and incubated with the nuclear stain Hoescht-33342 (Thermo Fisher scientific Inc., Waltham, MA, USA) for 20 minutes at room temperature, washed for 5 minutes in PBS and finally sealed with Vectashield HardSet antifade mounting medium (Vector laboratories, Inc. Burlingame, CA, 94010,

USA). A rat kidney sample was used as positive control. Images were obtained with a Confocal Laser Scanning Microscope SP5 (Leica Microsystems GmbH, Mannheim, Germany).

Immunoprecipitation

Four hundred µL-aliquots from every sample were incubated with the above described anti-AQP2 antibody at a final dilution of 1:40 (v:v) for 8 hours at 4ºC. After that, a Protein A–Agarose complex suspension (Pierce Biotechnology; Rockford, IL, USA) was added to the antigen-antibody complex and subsequently incubated for 2 hours with gentle mixing at 20ºC. After incubation, 200 µL of immunoprecipitation buffer (IP; 25 mM Tris, 150 mM NaCl, pH 7.2) were added, the sample was centrifuged for 3 minutes at 3000*g* at room temperature and the supernatant was discarded. This step was repeated six times following the manufacturer's instructions. After washing, elution of immunocomplexes was performed by incubation with 20 µL of 150 mM glycine-HCl buffer (pH 2.5) for 5 minutes at room temperature. Following incubation, samples were again centrifuged at 3000*g* for 3 minutes at room temperature and the resultant supernatant was collected. This step was repeated twice. Afterwards, the pH of the resultant eluate was adjusted by adding 4 µL of 1 M Tris buffer (pH 9.0). The finally obtained neutralized eluate was then processed through immunoblotting as described above.

Phosphorylation of tyrosine residues

Phosphorylation of tyrosine (TyrPhos) residues of AQP2 was evaluated in non-pregnant queens with low levels of progesterone (n=3), non-pregnant queens with high levels of progesterone (n=3), pregnant queens at D_{30} (n=3) and pregnant queens at D_{60} (n=3).

Western blotting analysis of AQP2 TyrPhos was performed in previously immunoprecipitated samples as described above. In this case, a rabbit anti-TyrPhos antibody was utilized at a dilution of 1:1,000 (v:v; PY-20; Sigma Aldrich, Sant Louis, Missouri, USA), whilst a secondary donkey anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc.; Dallas, TX, USA) was subsequently utilized at a dilution of 1:1,000. Membranes were exposed to radiograph films to reveal both AQP2 and PY-20 signals after 5 minutes of incubation with Inmobilon TM Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA). Integral densities of corresponding bands were analyzed through a computer-assisted image analysis system (Multi Gauge v 3.0 software system, Fujifilm, Tokyo, Japan).

Statistical analyses

Statistical analyses were conducted using a statistical software package (IBM SPSS Statistics for Windows 21.0, Chicago, IL, USA). Each queen was considered as an independent statistical case and all data were first checked for normality and homoscedasticity (i.e. homogeneity of variances) through Shapiro-Wilks and Levene tests, respectively. Because data satisfied parametric assumptions in all cases, a one-way analysis of variance (ANOVA) followed by post-hoc Sidak test were conducted with relative abundances of AQP2 and percentages of phosphorylated tyrosines in immunoprecipated AQP2 as independent variables. The queen stage (non-ovulated, ovulated or at different days pregnancy) was the factor.

The level of significance was set at $P \leq 0.05$, and data are shown as mean ± standard error of the mean (SEM).

Results

Immunoblotting

AQP2 expression was observed in both endometrium and transference zone samples in all the queens regardless the gestational age or the phase of sexual cycle (Fig. 1A). When the intensity of bands was quantified, uterus samples from non-pregnant queens with low levels of progesterone showed significantly (P<0.05) higher intensity of AQP2 than uterus samples from queens at 50 days of pregnancy (D_{50}) and the placental transference zone at days 30 (D_{30}) and 50 (D_{50}) of pregnancy (see Fig. 1B). No other significant difference between groups was observed. In addition, no significant correlation between the intensity of AQP2 expression and progesterone concentration was found (data not shown).

Immunohistochemistry

Uterine samples from both pregnant and non-pregnant queens showed an immunoreactive staining for AQP2 in myometrial cells and in both luminal and glandular epithelial cells of every stage evaluated (Fig. 2C-H). An apparent change in the AQP2-distribution related to serum levels of progesterone was observed. In effect, AQP2 was specifically located in the plasma membrane of both luminal and glandular epithelial cells in non-pregnant queens with low progesterone levels (Fig. 3A). In contrast, in pregnant queens and in non-pregnant animals with high progesterone levels, not only was AQP2 located in the plasma membrane but it was also present along the entire cytoplasm of both luminal and glandular epithelial cells (Fig. 3B). Finally, AQP2 immunoreactivity was also found in chorionic cells from the labyrinth and vascular

endothelium of pregnant queens in every pregnancy stage (Fig. 2I-L), without apparent differences among the studied gestational ages.

Immunoprecipitation and phosphorylation of tyrosine residues

No significant (P>0.05) differences in the tyrosine phosphorylation levels of AQP2 were observed in any of the evaluated tissues from pregnant individuals when compared with samples from non-pregnant queens (Fig. 4).

Discussion

The present study establishes for the first time the presence of AQP2 in both glandular and luminal uterine epithelium as well as in the transference zone in the domestic queen. Similar results have been previously described in several species such as human and bitch (Jablonski *et al.* 2003; Mobasheri *et al.* 2005; Aralla *et al.* 2009). In this way, our data suggest that the presence of uterine/placental AQP2 could be a common feature in a great number, if not all, mammals.

We found that the expression of AQP2 in the uterus is related to progesterone levels in serum. Again, these results agree with other studies conducted in other species (Jablonski *et al.* 2003; He *et al.* 2006). Indeed, the expression of AQP2 in the human endometrium has been found to be significantly higher during the secretory phase (high progesterone level) than during the proliferative phase (high estrogen level) of menstrual cycle (He *et al.* 2006). In the present study, neither differences between pregnant and non-pregnant queens nor between gestational ages were found in the relative AQP2 levels evaluated through Western blotting. In addition, no statistical correlation between serum levels of progesterone and AQP2 expression was observed. However, when immunohistochemistry results were evaluated, a progesterone-concomitant change in the distribution of AQP2 was observed across the cells. Indeed, while AQP2 was evenly distributed all over the cytoplasm and plasma membrane of luminal and glandular epithelium cells in pregnant and non-pregnant queens with high serum levels of progesterone, it was only found in the plasma membrane in non-pregnant queens with low levels of progesterone. These results would suggest that progesterone may be involved in the specific intracellular distribution of AQP2. Although data available at this moment are not enough to reach firm conclusion, it seems reasonable to suggest that progesterone could be involved in the preparation of

296 the endometrium for pregnancy. In this sense, Mathieu et al. (1989) demonstrated that
297 progesterone modulates the expression of calbindin-D_{9k} in the myometrium from both
298 pregnant and pseudo-pregnant rats. However, further research is needed to establish the
299 actual role of progesterone on the distribution and activity of AQP2.

300 Our results suggest that the observed localization changes of AQP2 were not
301 concomitant with significant variations in the percentages of TyrPhos of AQP2.
302 According to the literature, AQPs can be regulated by phosphorylation of Tyr and Ser
303 residues (Lu *et al.* 2008; Choi *et al.* 2014). Thus, phosphorylation of Tyr residues has
304 been described as a modulator of endocytosis and exocytosis of AQP2 (Katsura *et al.*
305 1997; He *et al.* 2006; Moeller *et al.* 2009), and vasopressin-induced changes of TyrPhos
306 levels in kidney AQP2 leads to activity changes of important signaling proteins, such as
307 adenylate cyclase and protein kinase A (Moeller *et al.* 2009). However, our data
308 indicate that changes in TyrPhos levels of AQP2 are not on the basis of the observed
309 progesterone-related distribution of uterine AQP2 during pregnancy. This result would
310 be in accordance with that observed in kidney, where vasopressin increases the AQP2
311 presence in the plasma membrane through a mechanism mediated by changes in the
312 phosphorylation of serine rather than tyrosine residues (Moeller *et al.* 2009).
313 Furthermore, the vasopressin-induced AQP2 localization changes in the kidney
314 collecting ducts are induced without significant phosphorylation changes in serine and
315 tyrosine residues of AQP2 (Arthur *et al.* 2015). At this moment, we can only speculate
316 regarding the nature of the mechanism/s underlying the observed AQP2 localization
317 changes. Therefore, while the present study points out that changes in the localization of
318 AQP2 could be related to progesterone levels, establishing the possible role of SerPhos
319 warrants further research.

320 The influence of progesterone on AQP2 localization during pregnancy could be
321 inferred by other data than the above discussed changes in subcellular distribution. In
322 this regard, we must remind that, according to the literature (Knospe *et al.* 2002), cat
323 blastocysts reach the uterus after 6-8.5 days of coitus. At that particular stage, embryos
324 are difficult to be detected at naked-eye due to their small size. This means that some of
325 the queens with high progesterone serum levels that were catalogued as non-pregnant
326 could have actually been pregnant. However, the presence or absence of pregnancy at
327 post-ovulation days would not affect the interpretation of the current results. In addition,
328 as implantation does not initiate until days 12-14 (Tsutsui and Stabenfeldt 1993), no
329 structural changes due to the possible presence of the placenta should be present before
330 12 days post-coitus.

331 Since the queen is an induced ovulator, the main mechanism responsible for the
332 LH surge leading to ovulation is the coitus. This implies that regardless of whether the
333 queen gets pregnant or not, serum levels of progesterone will increase beyond 2 ng/mL
334 approximately for 40 days, in the case of non-pregnant queens (pseudo-pregnant), and
335 60 days, in the case of pregnant queens (Johnston *et al.* 2001). In this manner, there
336 would not be hormonal differences between pregnant and non-pregnant queens during
337 the first days of diestrus when pregnancy is difficult to detect at a first glance. All these
338 facts would be in agreement with the hypothesis that progesterone is the responsible for
339 the observed changes in AQP2 expression.

340 Finally, it is noteworthy the presence of AQP2 in the labyrinth cells. This
341 presence suggests that AQP2 may take part in the supply of water for embryo
342 development during all pregnancy stages. Additionally, the presence of AQP2 in
343 labyrinth cells also suggests that one prominent role of uterine AQP2 during pregnancy
344 could be the control of embryo water disposal from uterus in accordance with Aralla *et*

345 *al.* (2012). Of course, further research is needed to determine the feasibility of this
346 hypothesis.

347 In conclusion, the present study has reported, for the first time, the presence of
348 AQP2 in uterus and placenta of queens. Moreover, our results suggest that AQP2 could
349 play an important, progesterone-modulated role during pregnancy in queens, through
350 regulating water metabolism of the uterus and control of fetal water intake through a
351 placental pathway.

352

References

1. Agre, P., King, L.S., Yasui, M., Guggino, W.B., Ottersen, O.P., Fujiyoshi, Y., Engel, A., Nielsen, S. (2002) Aquaporin water channels–from atomic structure to clinical medicine. J. Physiol. **542**, 3–16
2. Aralla, M., Borromeo, V., Groppetti, D., Secchi, C., Cremonesi, F., Arrighi, S., (2009) A colaboration of aquaporins handles water transport in relation to the estrus cycle in the bitch uterus, Theriogenology. **72**, 310-
3. Aralla, M., Mobasheri, A., Groppetti, D., Cremonesti, F., Arrighi S. (2012) Expression of aquaporin water channels in canine fetal adnexa in respect to the amniotic fluid production and absoption. Placenta **22**, 502-510
4. Arthur, J., Huang, J., Nomura, N., Jin, W.W., Li, W.L., Cheng, X., Brown, D., Ly, H.J. (2015) Characterization of the putative phosphorylation sites of the AQP2 C terminus and their role in AQP2 trafficking in LLC-PK_1 cells, Am. J. Physiol. Renal Physiol. **309**, F673-679
5. Bradford, M., (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry **72**, 248–256
6. Fresno, L., Rodriguez-Gil, J.E., Rigau, T., Pastor, J., Rivera del Alamo, M.M. (2012) Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. Placenta **33**, 522-7
7. Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Marumo, F., Sasaki, S. (1993) Cloning and expression of apical membrane water channel of rat kidney collecting tubule. Nature. **361**, 549–552
8. He, R.H., Sheng, J.Z.., Luo , Q., Jin, F., Wang, B., Qian, Y.L., Zhou, C.Y., Sheng, X., Huang, H.F. (2006) Aquaporin-2 expression in human endometrium correlates with serum ovarian steroid hormones. Life Sci. **79**, 423-9
9. Hildenbrand, A., Lalitkumar, L., Nielsen, S., Gemzell-Danielsson, K., Stravreus-Evers, A., (2006) Fertil. Steril. **86**, 1452-8
10. Jablonksi, E.M., McConnel, N.A., Hughes Jr., F.M., Huet-Hudson, Y.M. (2003) Estrogen regulation of aquaporins in the mouse uterus: Potential roles in uterine water movement. Biol. Reprod.**69**, 1481-7

11. Johnston, S.D., Root Kustritz, M.V., Olson, P.N.S. (2001) The feline estrous cycle. In: Canine and Feline Theriogenology. (Eds Saunders) p402
12. Katsura, T., Gustafon, C.E., Ausiello, D.A., Brown, D. (1997) Protein kinase A phosphorylation is envolved in regulated exocitosis of aquaporin-2 in transfected LLC-PK1 cells, Am. J. Physiol. **272**, F817-F822
13. Knospe, C. (2002) Periods and stages of the prenatal development in the domestic cat. Anat. Histol. Embryol. **31**, 37-51
14. Lu, H.J., Matsuzaki, T., Bouley, R., Hasler, U., Qin, Q.H., Brown, D. (2008) The phosphorylation state of serine 256 is dominant over serine 261 in the regulation of AQP2 trafficking in renal epithelial cells. Am. J. Physiol. **272**, F290-F294
15. Mathieu, C.L., Burnett,S.H., Mills, S.E., Overpeckt, J.G., Bruns, D.E., Bruns. M. E., (1989) Gestational changes in calbindin-D9k in rat uterus, yolk sac, and placenta: implications for maternal-fetal calcium transport and uterine muscle function, Proc. Natl. Acad. Sci. USA **86**, 3433-3437
16. Miglino, M.A., Ambrósio, C.E., dos Santos Martins, D., Wenceslau, C.V., Pfarrer, C., Leiser, R.(2006) The carnivore pregnancy: the development of the embryo and fetal membranes. Theriogenology. **66,** 1699-702.
17. Mobasheri, A., Wray, S., Marples, D. (2005) Distribution of AQP2 and AQP3 water channels in human tissue microarrays. J. Mol. Hist. **31**, 1-14
18. Moeller, H. B. Praetorius, J. Rützler, M.R., Fenton, R.A. (2009) Phosphorylation of aquaporin-2 regulates its endocytosis and protein-protein interactions. Proc. Natl. Acad. Sci. USA. **107**, 424-9
19. Nejsum, L.N., Zelenina, M., Aperia, A., Frøkiarer, J., Nielsen, S., (2005) Bidirectional regulation of AQP2 trafficking and recycling: Involvement of AQP2-S256 phosphorylation. Am. J. Physiol. Renal Physiol. **288**, F930-F938
20. Park, E., Kwon, T. (2015) A minireview of Vasopresin-regulated Aquaporin-2 in kidney collecting duct cells. Electrolyte Blood Press **131**, 1-6
21. Richard, C., Gao, J.,Brown, N., Reese, J. (2003) Aquaporin water channel genes are differentially expressed and regulated by ovarian steroids during the periimplantation period in the mouse. Endocrinology. **144**, 1533–1541.
22. Sasaki, S., Kuwahara, M., Yamashita, Y., Marumo, F. (2000) Structure and function of AQP2. Nephrol. Dial Transplant. **15**, (Suppl 6) 21–22.

23. Sirois, J., Doré, M., (1997) The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process, Endocrinology. **138**, 4427–4434

24. Stevens, A.L., Breton, S., Gustafson, C.E., Bouley, R., Nelson, R.D., Kohan, D.E., Brown, D. (2000) Aquaporin 2 is a vasopressin-independent, constitutive apical membrane protein in rat vas deferens. Am. J. Physiol. Cell Physiol. **278**, C791–C802

25. Tsutsui, T., Stabenfeldt, G.H. (1993) Biology of ovarian cycles, pregnancy and pseudopregnancy in the domestic cat. J. Reprod. Fertil. Suppl. **47**, 29-35

26. Woo, J.S., Wan, C. W., Cho, K.M. (1985) Computer-assisted evaluation of ultrasonic fetal weight prediction using multiple regression equations with and without the fetal femur length, J. Ultrasound Med. **4,** 65-7.

Fig. 1. Western blotting expression for APQ-2. **A.** Radiograph films showing a 29 KDa bands compatible with AQP2. Note that the expression is present in all the evaluated groups. MW: molecular weight marker; LP: non-pregnant queens with low levels of serum progesterone; HP: non-pregnant queens with high levels of serum progesterone; UT: uterus; TZ: transference zone; D30: 30 days of pregnancy; D40: 40 days of pregnancy; D50: 50 days of pregnancy; D60: 60 days of pregnancy. No statistically significant difference among the different groups was observed. **B.** Relative AQP2 abundance calculated by means of the Multi Gauge v3.0 software system. Statistically significant differences are indicated with different superscripts.

Fig. 2. Immunohistochemistry images for AQP2 expression. Cellular nuclei are blue-stained with DAPI dye, while AQP2 is red-stained. **A:** Negative control in a uterine sample; **B:** Positive control in a rat kidney sample. Note the red mark for AQP2 around the collecting ducts membrane of the kidney; **C:** Uterine sample from a non-pregnant queen with low serum levels of progesterone. Note the red mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **D:** Uterine sample from a non-pregnant queen with high serum levels of progesterone. Note the red mark in the cytoplasm of the cells from the glandular and luminal epithelia (white arrows); **E:** Uterine sample from D30 pregnancy. Note the red mark in the cytoplasm of the myocytes from the myometrium (white asterisk) and the cells from the endometrial glands (white arrow); **F:** Uterine sample from D40 pregnancy. Note the red mark in the cytoplasm of the myocytes from the myometrium (white asterisk) and the cells from the endometrial glands (white arrow); **G:** Uterine sample from a D50 pregnancy. Note the red mark in the cytoplasm of the cells from the luminal epithelium (white arrow); **H:** Uterine sample from a D60 pregnancy. Note the

red mark in the cytoplasm of the cells from the luminal epithelium (white arrow); **I:** Sample from the transference zone (TZ) in a D30 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); **J:** Sample form the TZ in a D40 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); **K:** Sample from the TZ in a D50 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); **L:** Sample from the TZ in a D60 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow).

Fig. 3. **A:** Immunohistochemistry image from a non-pregnant queen with low levels of progesterone (LP). AQP2 (red mark) is distributed only in the cellular membrane of the cells from the glandular and luminal epithelium (white arrow). **B:** Immunohistochemistry image from a pregnant queen (D50). AQP2 is evenly distributed all over the cellular cytoplasm, not just the membrane, in both the glandular (white arrow) and luminal epithelia (white asterisk).

Figure 4. **A:** Western blotting for AQP2 after performing immunoprecipitation. **B**: Western blotting for PY-20 of the same samples after performing immunoprecipitation. LP: non-pregnant queens with low levels of serum progesterone; HP: non-pregnant queens with high levels of serum progesterone; UT: uterus; TZ: transference zone; D30: 30 days of pregnancy; D60: 60 days of pregnancy. **C:** Graph representing the proportion between AQP2 and its phosphorylated form. No statistically difference was observed.

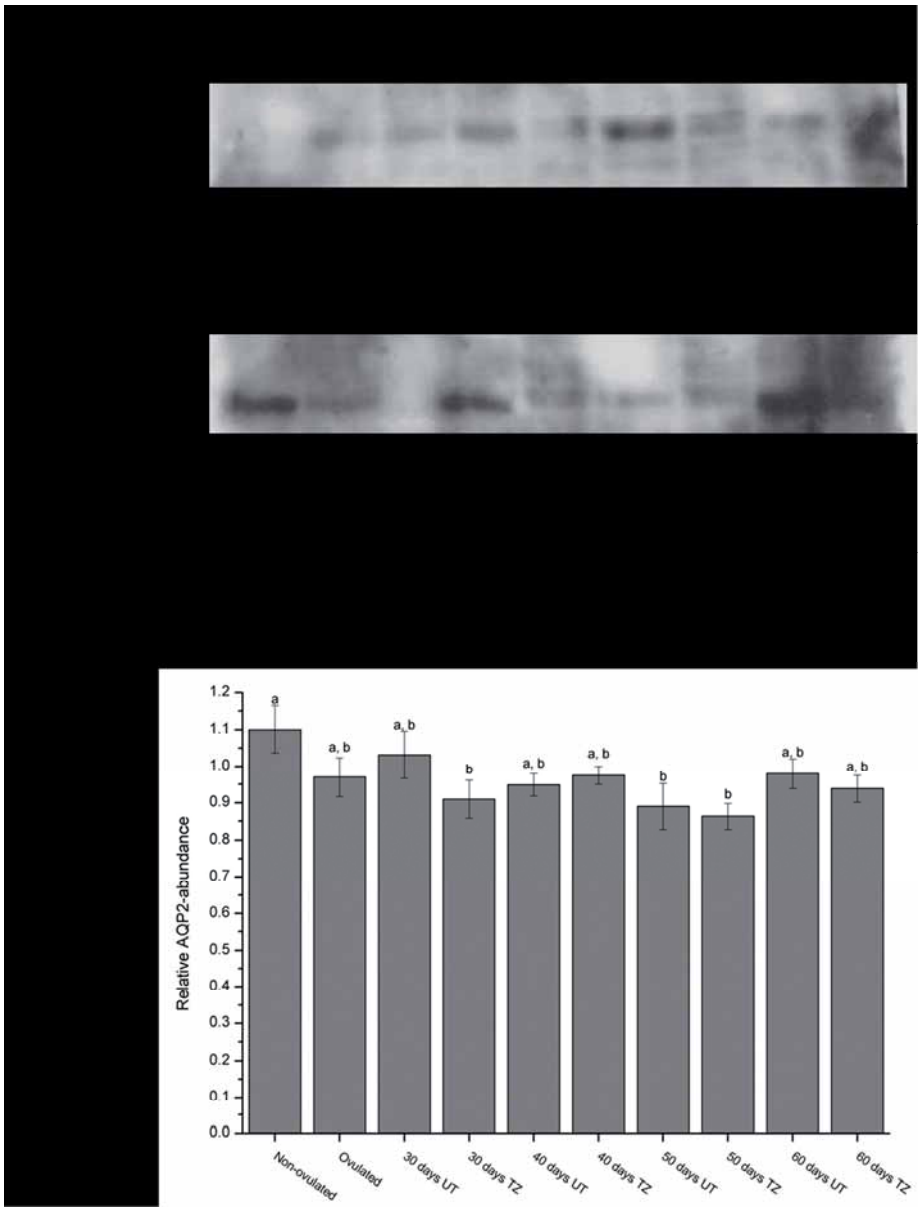


Fig. 1. Western blotting expression for APQ-2. A. Radiograph films showing a 29 KDa bands compatible with AQP-2. Note that the expression is present in all the evaluated groups. MW: molecular weight marker; LP: non-pregnant queens with low levels of serum progesterone; HP: non-pregnant queens with high levels of serum progesterone; UT: uterus; TZ: transference zone; D30: 30 days of pregnancy; D40: 40 days of pregnancy; D50: 50 days of pregnancy; D60: 60 days of pregnancy. No statistically significant difference among the different groups was observed. B. Relative AQP-2 abundance calculated by means of the Multi Gauge v3.0 software system. Statistically significant differences are indicated with different superscripts.

180x237mm (150 x 150 DPI)

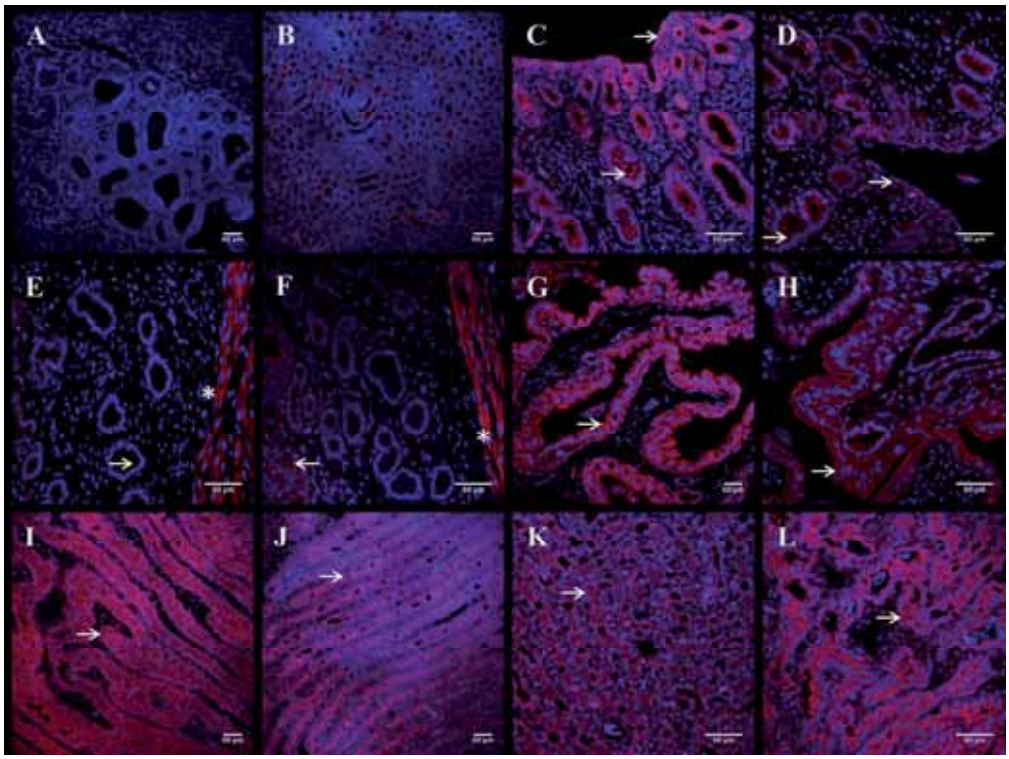


Fig. 2. Immunohistochemistry images for AQP-2 expression. Cellular nuclei are blue-stained with DAPI dye, while AQP-2 is red-stained. A: Negative control in a uterine sample; B: Positive control in a rat kidney sample. Note the red mark for AQP-2 around the collecting ducts membrane of the kidney; C: Uterine sample from a non-pregnant queen with low serum levels of progesterone. Note the red mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); D: Uterine sample from a non-pregnant queen with high serum levels of progesterone. Note the red mark in the cytoplasm of the cells from the glandular and luminal epithelia (white arrows); E: Uterine sample from D30 pregnancy. Note the red mark in the cytoplasm of the myocytes from the myometrium (white asterisk) and the cells from the endometrial glands (white arrow); F: Uterine sample from D40 pregnancy. Note the red mark in the cytoplasm of the myocytes from the myometrium (white asterisk) and the cells from the endometrial glands (white arrow); G: Uterine sample from a D50 pregnancy. Note the red mark in the cytoplasm of the cells from the luminal epithelium (white arrow); H: Uterine sample from a D60 pregnancy. Note the red mark in the cytoplasm of the cells from the luminal epithelium (white arrow); I: Sample from the transference zone (TZ) in a D30 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); J: Sample form the TZ in a D40 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); K: Sample from the TZ in a D50 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow); L: Sample from the TZ in a D60 pregnancy. Note the red mark in the cytoplasm of the cells from the labyrinth (white arrow).

248x185mm (150 x 150 DPI)

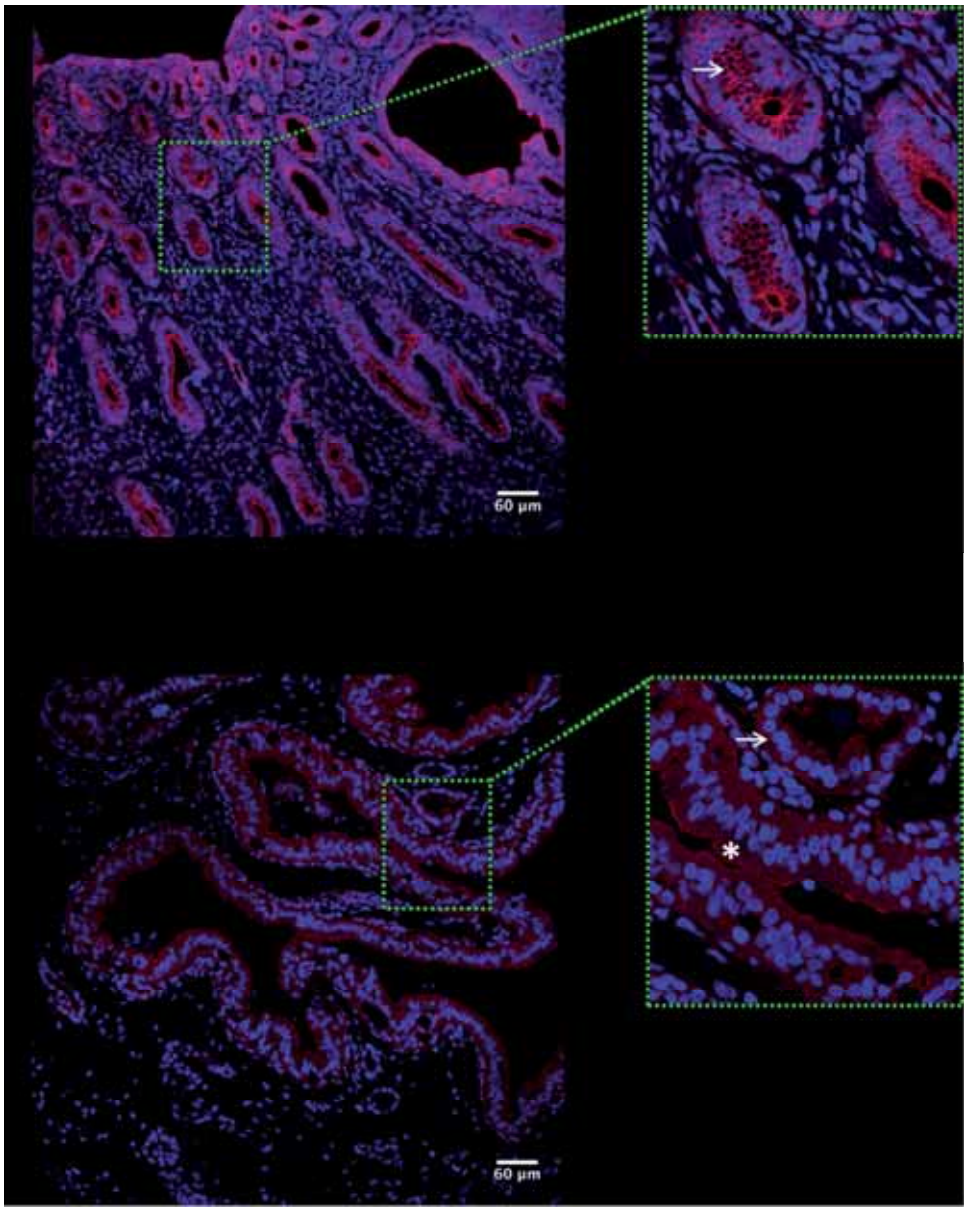


Fig. 3. A: Immunohistochemistry image from a non-pregnant queen with low levels of progesterone (LP). AQP-2 (red mark) is distributed only in the cellular membrane of the cells from the glandular and luminal epithelium (white arrow). B: Immunohistochemistry image from a pregnant queen (D50). AQP-2 is evenly distributed all over the cellular cytoplasm, not just the membrane, in both the glandular (white arrow) and luminal epithelia (white asterisk).

180x225mm (150 x 150 DPI)

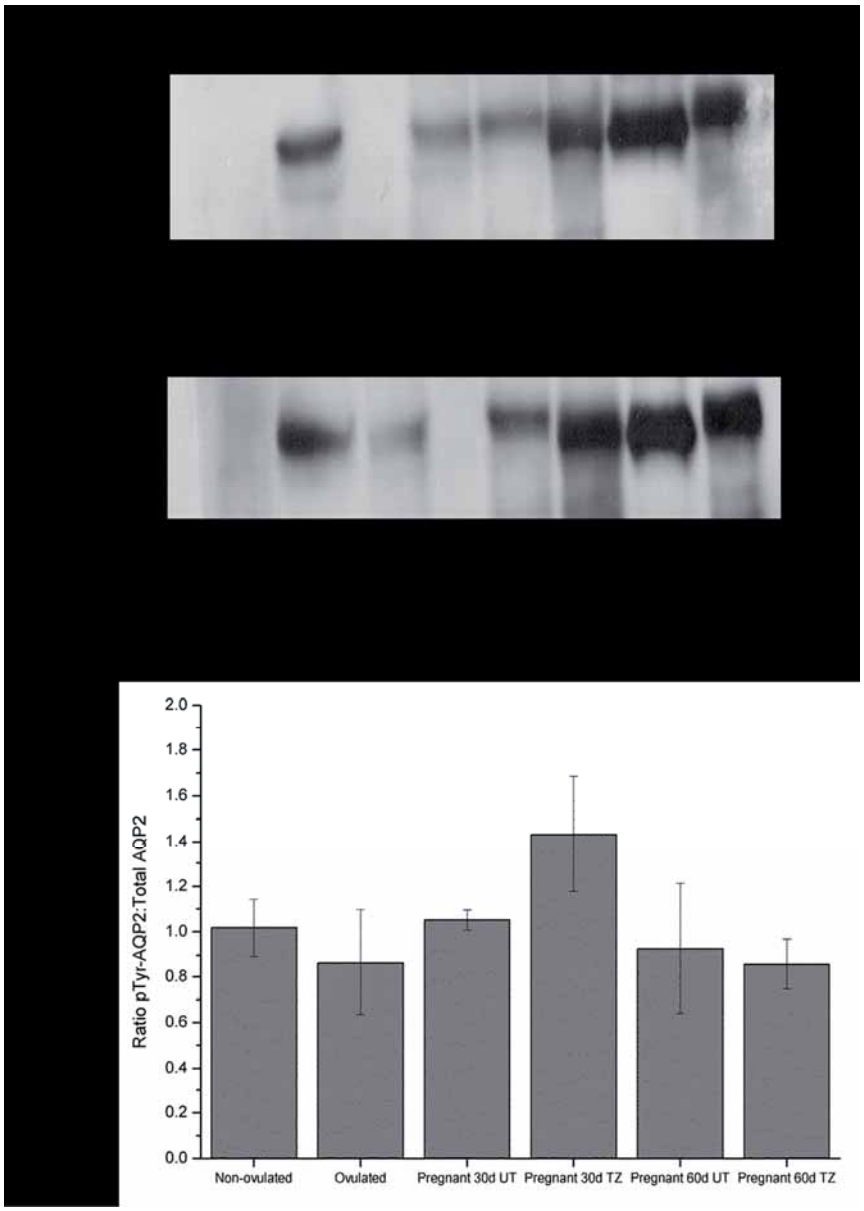


Figure 4. A: Western blotting for AQP-2 after performing immunoprecipitation. B: Western blotting for PY-20 of the same samples after performing immunoprecipitation. LP: non-pregnant queens with low levels of serum progesterone; HP: non-pregnant queens with high levels of serum progesterone; UT: uterus; TZ: transference zone; D30: 30 days of pregnancy; D60: 60 days of pregnancy. C: Graph representing the proportion between AQP-2 and its phosphorylated form. No statistically difference was observed.

173x243mm (150 x 150 DPI)

Article 3: Glucose transporter 1 and 3 expression and location throughout different sexual and pregnancy stages in queen reproductive tract: A immunoblotting and confocal microscope study.

Glucose transporter 1 and 3 expression and location throughout different sexual and pregnancy stages in the queen reproductive tract: An immunoblotting and confocal microscope study.

L. Ferré-Dolcet[1], M. Yeste[2], M. Vendrell[1], T. Rigau[1,] JE. Rodríguez-Gil[1] MM. Rivera del Alamo[1*]

[1]*Animal Medicine and Surgery Department, Veterinary College, Universitat Autònoma de Barcelona, Bellaterra, 08193, Spain*

[2]*Department of Biology, University of Girona, Girona, E-17071 Girona, Spain*

**Corresponding author: VO-229, Departament de Medicina i Cirurgia Animals, Facultat de veterinària, Edifici V, Campus Universitari, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain. Tel.: +34 935811045.*

E-mail addresses: mariamontserrat.rivera@uab.cat

Keywords: Glucose transport, Queen, Endometrium, Placenta, Trophoblasts, GLUT

Abstract

Adequate glucose transfer from mother to fetus is crucial for maintaining the pregnancy and for an appropriate development of the fetus. The facilitative glucose transporters (GLUTs) are responsible for the glucose transport across the cellular membranes. The present study, the expression of GLUTs 1 and 3 isoforms were investigated in both uterus and placental transference zone through different sexual and pregnancy phases in the queen using immunoblotting and immunohistochemistry techniques. Both GLUT1 and GLUT3 were expressed in both uterus and placental transference zone in every sample evaluated without significative differences on its expression. Immunohistochemistry showed expression of GLUT1 and 3 in both uterine glandular and luminal epithelia. In addition, GLUT1 was expressed also in the myometrium and vasculaer endothelia. In the transferenze zone of pregnant queens, GLUT1 showed a distribution on the apical and basolateral membrane of the trophoblastic cells of the chorion while GLUT3 was present in both cytoplasm and cell membrane of the chorionic layer. In conclusion, GLUTs 1 and 3 are responsible for glucose uptake in both uterine and placental tissues for fetus development.

Introduction

Glucose metabolism is important to prepare the epithelium and the stroma for embryo implantation and also for the proper differentiation of the functionalis layer in order to support the developing conceptus (Von Wolff *et al.,* 2003, Frolova *et al.*, 2009, Kim and Moley 2009, Frolova and Moley 2011)..

Due to it is a polar molecule, glucose is not soluble in plasma membrane and has to be transported by proteins across it to reach the cytoplasm (Bell *et al.,*, 1990). These carrier transporter proteins are named glucose transporters and are divided in two groups depending if are Na+/Glucose co-transporters (SGLTs) or facilitative diffusion glucose transporters (GLUTs) (Bell *et al.,*, 1990).

The function of these transporters is to maintain euglycemia controlling the glucose uptake into the cells and releasing it from the liver when circulating glucose levels decrease (Hediger *et al.,* 1994). These transporters are also responsible for glucose reabsorption from glomerular filtrate (Hediger *et al.*, 1994).

SGLTs allow glucose influx through the luminal membrane and GLUTs allow glucose efflux through the basolateral membrane (Sabino-Silva *et al.,* 2010). This is important for trans-epithelial glucose transport in small intestine cells, lungs, liver, renal proximal tubules and salivary gland ducts (Lee *et al.,* 1994).

The facilitative transporters (GLUT) utilize the diffusion gradient of glucose and other sugars across the plasma membranes and exhibit different substrate specificities, kinetic properties and tissue expression profiles (Wood and Trayhurn, 2003). GLUTs are proteins of around 500 amino acids and their family members can be divided depending on the sugar they transport (Joost and Thorens, 2001). Several GLUTs have been identified in the endometrial stroma of human (von Wolff *et al.*, 2003), mice (Frolova

and Molley, 2011) and rat endometrium (Korgun *et al.*, 2001), but little information is available. On the other hand, of the 14 GLUTs described in the literature, only four have been described so far to be expressed in the placental layers (Shin *et al.*, 1997, Illsey 2000, Limesand *et al.*, 2004, Wooding *et al.*, 2000, Wooding *et al.*, 2005). In addition to expression studies, recent studies have also demonstrated that steroid hormones are involved in the glucose metabolism regulation by regulating these glucose transporters expressions (Kuo *et al.*, 2015).

GLUT1 was the first glucose transporter to be described (Mueckler *et al.*, 1985). It is a very ubiquitous protein and the most abundant in the endothelial cells (Frolova and Molley, 2011). It is considered the major contributor to glucose homeostasis due to its high affinity for glucose but low affinity for fructose (Carruthers, 1990). On the other hand, GLUT3 has been described to have a highest affinity for glucose than other hexoses, such as mannose, galactose and xylose (Simpson *et al.*, 2008). However, it does not transport fructose. It was originally designated as a neuronal glucose transporter because of its high expression in neurons (Nehlig A. 1996). Several studies have demonstrated that GLUT3 plays an important role in reproductive system and its expression has been described in human and rat sperm (Haber *et al.*, 1993), embryo (Pantaleon *et al.*, 1997), male and female reproductive tract and placenta (Hahn D. *et al.*, 2001).

The objective of the present study was therefore to identify and describe the expression and localization of both GLUT1, and -3 in the queen endometrium and placenta to establish if they are involved in pregnancy development, as well as matching its possible correlation with serum progesterone levels.

Materials and methods

Samples Collection and processing

Thirty-eight stray queens belonged to a program of sterilization of stray-cats at the Universitat Autònoma de Barcelona (Spain) were included in the present study. For the inclusion, negative tests to feline leukemia (FeLV) and feline immunodeficiency (FIV) and no sign of illness were mandatory parameters. Sexual stage of the queens was not relevant for the inclusion or exclusion in the study.

Queens were premedicated with ketamine (5 mg/kg, Imalgene 1000, Laboratorios Merial, Barcelona, Spain), buprehorphine (20 μg/kg, Buprecare, Divasa-Farmavic S.A, Gurb, Spain) and midazolam (0.2 mg/kg, Midazolam Normon, Laboratorios Normon S.A., Madrid, Spain) IM. Anesthesia was performed with IV propofol (4 mg/kg, Vetofol, Laboratorios Esteve, Barcelona, Spain) and anesthetic status was maintained with 1.5-2% isoflorane (Isoflo, Laboratorios Esteve, Barcelona, Spain).

Routine ovariohysterectomy was performed by midline laparotomy. Once removed, uteri were examined macroscopically in order to determine the presence or absence of pregnancy. On the one hand, non-pregnant females were distributed into two groups, ovulated (n=7) and non-ovulated (n=7), according to serum progesterone levels (see below). On the other hand, pregnant queens were divided according to their gestational age (Knospe, 2002, Fresno *et al.,* 2012) into 30 (D_{30}; n = 8), 40 (D_{40}; n = 5), 50 (D_{50}; n = 4) and 60 (D_{60}; n = 7) days of pregnancy.

Uterine full-thickness biopsies were obtained from every queen. In addition, biopsies from the placental transfer zone from pregnant queens were also obtained. Every biopsy was split in two samples. One was kept frozen at -80ºC for Western blotting analyses,

while the other was fixed in a 10% paraformaldehyde during 48h for immunohistochemistry evaluations. In the case of uterine samples for Western blotting analyses, the endometrium was removed from the myometrium with a scalpel and kept for the analyses.

The sampling collection was performed under the guidelines of the Ethical Committee Animal Care and Research, Autonomous University of Barcelona (CEEAH, code 2939).

Progesterone concentration

A blood sample from the jugular vein was obtained from every queen previously to surgery. Blood samples from the jugular vein were allowed to clot at room temperature in glass tubes and centrifuged at 2500*xg* for 5 minutes. Serum was collected and kept frozen in Eppendorf tubes at -20ºC until progesterone analysis was performed (Immulite® 1000, Siemens Healthcare Diagnostics, Cornellà del Llobregat, Spain).

According to serum progesterone levels, non-pregnant queens were split into non-ovulated or low serum progesterone level (progesterone concentration $\leq$ 1 ng/mL) and ovulated or high serum progesterone level (progesterone concentration $\geq$ 2 ng/mL) (Fresno *et al.*, 2012). Queens with values between 1 and 2 ng/mL were excluded from the study.

Western blotting

First of all, total protein concentration from each sample was determined by using the Braford technique (Bradford, 1976). For that purpose, samples were previously homogenized in 1 mL of protein extraction buffer (50mM TRIS HCl pH 7,4, 1mM EDTA, 10mM EGTA, 25mM DTT, 1.5% Tritonx100, 1mM PMSF, 10μg/mL Leupeptine, 1mM Ortovanadate, 1mM Benzamidine) with an Ultra-Turmax T25 basic

homogenizer (IKA-WERKE, Staufen, Germany). Supernatants were collected after centrifuging the samples at 4ºC at 2500g during 5 minutes and kept frozen at -80ºC until Western blotting analysis was performed.

Separation of proteins was then performed by SDS-PAGE electrophoresis in 10% (w/v) acrylamide gels (Sirois and Dore, 1997). After that, proteins were transferred to nitrocellulose membranes and Ponceau S dye at 0.1% (w/v) was used to confirm that the transference was successful. To avoid unspecific unions, nitrocellulose membranes were blocked in a 2% BSA (w/v) solution. For GLUT1 and GLUT3 detection, anti-GLUT1 and anti-GLUT3 monoclonal primary anti-rabbit antibodies (Sigma-Aldrich, 3050 Spruce Street, Saint Louis, MO 63103 USA) at 1:1000 (v:v) dilution were used. Membranes were then incubated at 4ºC overnight.

Membranes were then washed in a TBST solution (Tris buffered saline, 0,1%, tween20) 3 times for 5 minutes to remove the excess of primary antibody. After washing, membranes were exposed to an anti-rabbit secondary antibody (Santa Cruz Biotechnology Inc., 10410 Finnell Street, Dallas, Texas 75220, USA) for 45 minutes at a 1:1000 (v:v) dilution.

Membranes were then washed again 6 times for 10 minutes with TBST and then incubated 5 minutes with Inmobilon TM Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA 01821, USA). Then, membranes were exposed to radiograph films to reveal the GLUTs bands (50-60 KDa).

After the evaluation of GLUTs expression, membranes were immersed in a stripping buffer (10 gr glycine, 1 gr SDS, 10 ml Tween 20, pH 2.2, in 1 L of pure water) for specific GLUTs marking removal. Membranes were then re-probed with an anti-mouse α-tubulin antibody at a 1:1000 dilution (ABR Affinity BioReagents, CO, USA) to verify that the same amount of protein for every sample was loaded.

Specific GLUTs bands densities were analyzed with a computer-assisted image analysis system (Multi Gauge v 3.0 software system, Fujifilm, Tokyo, Japan).

Immunohistochemistry

Biopsy samples were embedded in paraffin blocks and 4 μm-thick sections were obtained. For dewaxing, sections were immersed two times in Xilene during 5 minutes. After that, sections were hydrated by immersing twice during 5 minutes in a descending concentration of alcohols (100%, 96% 70% respectively) and washed twice in PBS (1x) for 5 minutes with slight agitation. This process was performed at room temperature. For antigen retrieval, sections were immersed into a citrate buffer (10mM sodium citrate, 0.05% Tween20, pH=6.0) for 20 minutes at 96ºC and rinsed twice in PBS during 5 minutes. Immersions of 1 hour into a 2% BSA solution at room temperature were performed for blocking unspecific antibody reactions. After blocking, samples were incubated with the corresponding primary antibody overnight at 4ºC at 1:100 dilution (v/v). Afterwards, sections were washed 6 times for 10 minutes in PBS and then, incubated 3 hours with a secondary anti-mouse Alexa Fluor 568-linked antibody at 4ºC. Nuclear staining was performed with a 20 minutes with Hoescht-33342 at room temperature (Thermo Fisher scientific Inc. Waltham, MA 02451, USA). A Vectashield HardSet antifade mounting medium (Vector laboratories, Inc. Burlingame, CA, 94010 USA) was used for sealing treated sections. Every sample was evaluated with a Confocal microscope SP5 (Leica Microsystems GmbH, Mannheim, Germany).

Statistical Analysis

Analyses of results were conducted with a statistical package (SPSS for Windows, Ver. 21.0; Chicago, IL, USA). Data were checked through normality

(Shapiro-Wilk test) and homogeneity of variances (Levene test). As data were not distributed normally, they were transformed through arcsin √x. However, this transformation did not correct the distribution and non-parametric tests were used as an alternative to ANOVA. Therefore, Kruskal-Wallis test was used as a non-parametric ANOVA and this was followed by Mann-Whitney test for pair-wise comparisons. Dependent variables were the relative abundances (i.e. normalizad against β-tubulin) of GLUT1 and GLUT3.

Spearman correlations were calculated between P4 levels and relative abundances of GLUT1 and GLUT3. The level of significance was set at $P<0.05$ and data are shown as mean ± standard error of the mean.

Results

Western blotting analyses

Cells from both luminal and glandular epithelium, as well as chorionic layers from the transplacental zone, showed specific bands of 55 and 60 KDa, corresponding to GLUT1 and 3 respectively.

When the intensity of the bands was analyzed, no statistically significant ($P>0.05$) differences among the six groups were observed regardless their gestational age or sexual cycle phase of the queen (Table X).

On the other hand, GLUT3 showed a negative significant correlation ($P<0.05$) with serum progesterone levels, with an r value of -0,37 (Table X), while GLUT1 showed no correlation (data not shown).

Inmunohistochemistry analyses

Non-pregnant queens with low concentrations of serum progesterone showed an immunofluorescence pattern against both GLUT1 and GLUT3. GLUT1 was expressed in the cytoplasm membrane in both laminal and glandular epithelia, in the vascular endothelia of the endometrium and myometrium. On the other side, GLUT3 was expressed in the apical plasma membrane of both laminar and glandular epithelia, but not in the vascular endothelia of the endometrium.

In non-pregnant queens with high levels of progesterone, both glucose transporters GLUT1 and 3 showed a homogeneous pattern of fluorescence in the cytoplasm of both laminar and glandular epithelia in addition to cell membrane. Moreover, myometrial cells expressed also fluorescence against both GLUTs. At the vascular endothelia level, only GLUT1 showed immunofluorescence.

In the pregnant queens, GLUT1 was homogeneously expressed in the cytoplasm and the cell membrane of both glandular and laminar epithelial cells, myometrial cells and vascular endothelia regardless their gestational age. When transference zone was evaluated, GLUT1 showed immunoreactivity at the apical and basolateral membrane of the syncytiotrophoblastic and cytotrophoblastic cells of the chorionic labyrinth in every stage evaluated. Fetal endothelia showed no expression for GLUT1.

On the other side, GLUT3 was present in the cytoplasm of both glandular and laminar epithelial cells, myometrial cells and vascular endothelia regardless their gestational age. When transference zone samples were evaluated, GLUT3 immunoreactivity was present in the syncytiotrophoblastic and cytotrophoblastic cells of the chorionic labyrinth with a homogeneous pattern of distribution in both membrane and cell cytoplasm. In addition to maternal endothelia, also fetal vessels expressed GLUT3.

Discussion

In the present study, data concerning to facilitative glucose transporters GLUT1 and GLUT3 in the queen endometrium and transplacental zone at different sexual and pregnancy stages are provided. According to the results, both GLUTs may be involved in the facilitate glucose transfer to the fetus. Previous studies have demonstrated the presence of GLUT 1 and 3 in human placenta (Bell *et al.*, 1990, Shepard *et al.*, 1992) and rat endometrium and placenta (Yamaguchi *et al.*, 1996). Nevertheless, not so much information is available about these transporters and its possible function in reproductive system.

Yamaguchi *et al.* (1996) and Frolova *et al.* (2009) previously described that GLUT1 increases during fetal development and has a positive correlation with serum progesterone levels in rat and human respectively. However, our results are in disagreement. No correlation between GLUT1 expression in the endometrium or placental transference zone and serum levels of progesterone has been observed in the present study. A possible explanation could be the fact that mouse and human have a hemochorial placenta, while queens present an endotheliochorial placenta. So, maybe the fact of presenting different types of placenta, with different degrees of invasiveness would lead to a different mechanism of action.

On the other hand, Frolova and Moley (2011) described that GLUT1 was the most abundant glucose transporter in murine and human endometrium. However, in our study no differences between the expression of GLUT1 and GLUT3 along the pregnancy were observed, suggesting that both have a similar role in the glucose transport from the dam to the fetus. As it has been previously mentioned, GLUT3 has a high affinity for glucose, but its able to transport mannose, xylose and galactose (Simpson *et al.*, 2008).

With this on mind, we can suggest that at the queen endometrium, not only the glucose uptake is necessary, but also different kinds of hexoses.

A previous study (Wooding *et al.,* 2007) has already described the presence of GLUT1 in the queen placenta in different days of pregnancy, but not in the endometrium from both pregnant and non-pregnant queens These results do not match with what we describe in our study, where uterine layers of every stage evaluated presented a fluorescence pattern in both luminal and glandular epithelia. Our results suggest that uterine uptake of glucose may be important to create a glucose influx from the mother endometrium to the placenta and the growing fetus.

In addition to endometrial epithelia, GLUT1 was also present in the maternal capillaries. These results are in disagreement with previous studies performed in queens (Wooding *et al.,* 2007), which did not observe endothelial expression for GLUT1. These differences on GLUT1 expression may be due to the fact of using newest antibodies with high sensitivity to GLUT1 protein. Moreover, the innovation of techniques in both immunoblotting and immunohistochemistry may be determinant in detection of this protein. With our results by hand, it is not difficult to think that glucose uptake by the fetus should enter by a hematological way from the maternal capillaries.

Regarding to the expression of GLUT3 in the placenta, studies have shown variable results. Thus, while Esterman et al. (1997) did not observed the presence of GLUT3 in the human placenta, Illsley *et al.* (1998) and Ogura *et al.* (2000) detected GLUT3 in the first trimester of mouse and human trophoblastic cells suggesting that this GLUT may be important in the early postimplantational period when glucose uptake should be constant. Both authors suggested that the expression of GLUT3 in the extra-embryonic layers would permit maternal glucose to travel to the demanding embryo.

Regarding to progesterone concentration, GLUT3 showed a negative correlation with progesterone showing that lower levels of progesterone note a higher expression of this protein. Moreover, non-ovulated queens seemed to express more GLUT3 than ovulated queens. These results can suggest that at time of implantation, other transporters in addition to GLUT3 may be involved in the preparation for embryo implantation, where glucose requirements may be increased. In addition, non-pregnant queens with low levels of progesterone and pregnant queens at D60 express more GLUT3 in both endometrium and placental transference zone. These results are in disagreement with previous studies which demonstrate that GLUT3 was only present during the first pregnancy trimester of mouse and human placenta (Esterman *et al.*, 1997, Hahn *et al.*, 2001, Simpson *et al.*, 2008). Once again, the results suggest that differences between species or types of placenta could determine the mechanism responsible for glucose transport from the dam to the fetus. Our immunohistochemical results showed that vascular endothelia only expressed GLUT3 in the chorionic membrane of pregnant queens but not in the endometrium of both pregnant and non-pregnant queens. Brown *et al.*, (2011) also described that GLUT3 was present in the vascular endothelia form human placenta from the first trimester of pregnancy on. These results suggest that GLUT3 may be a critical mediator of glucose uptake from the maternal circulation to the trophoblastic cells and the energetically demanding fetus.

In agreement with previous studies, confirmation of our observations of GLUT3 at queen placental membranes is very important. As some groups mentioned above described no presence of GLUT3 from second trimester of pregnancy and above, our study is completely disagreeing, showing a clear positive staining in trophoblastic cells of the chorionic layer across all the queen pregnancy. As it has been stated before, queen placentation totally differs from mouse and human placentation. Queen placenta

is an endotheliochorial placenta, meaning that chorionic layer invades the endometrial epithelium in order to contact directly to the maternal vascularization. Moreover, it is considered a zonary placenta. In the other side, mouse and human discoid placenta is also considered as a hemochorial placenta, where fetus contacts directly with maternal blood circulation. These characteristics may be important for supporting our results, contrasting the fact that in the endotheliochorial placentas, transport from solutes from the mother circulation should be done until delivery. In conclusion, the presence of these transporters in the placental membranes suggests an implication in hexose uptake and movement across membranes from mother to fetus.

References:

Barret MP, Walmsley AR, Gould GW (1999) Structure and function in facilitative sugar transporters. Curr Opinion Cell Biol 11:496-502

Bell GI, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, *et al.*, Molecular biology of mammalian glucose transporters. *Diabetes Care* 1990; 13: 198-208.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248-256.

Brown K, Heller DS, Zamudio S, Illsley NP (2011) Glucose transporter 3 (GLUT3) protein expression in human placenta across gestation. Placenta 32: 1041-1049.

335 Carruthers A (1990) Facilitated difussion of glucose. Physiol Rev 70:1135-76

336

337 Douard V, Ferraris RP. Regulation of the fructose transporter GLUT5 in health and
338 disease. *Am J Physiol Endocrinol Metab* 295: E227–E237, 2008.

339

340 Esterman A, Greco MA, Mitani Y, Finlay TH, Ismail-Beigi F, Dancis J (1997) The
341 effect of hypoxia on human trophoblast in culture: morphology, glucose transport and
342 metabolism. Placenta 18: 129-136.

343

344 Fresno L, Rodriguez-Gil JE, Rigau T, Pastor J, Rivera del Alamo MM.
345 (2012) Modulation of the biochemical composition of amniotic and allantoic fluids as a
346 control mechanism of feline foetal development. Placenta **33**, 522-7

347

348 Frolova A, Flessner L, Chi M, Kim ST, Foyouzi-Yousefi N, Moley KH. (2009)
349 Facilitative glucose transporter type 1 is differentially regulated by progesterone and
350 estrogen in murine and human endometrial stromal cells. Endocrinology. 150(3):1512-
351 20.

352

353 Frolova AI, Moley KH. (2011) Quantitative analysis of glucose transporter mRNAs in
354 endometrial stromal cells reveals critical role of GLUT1 in uterine receptivity.
355 Endocrinology. 152(5):2123-8.

356

357 Haber RH, Wienstein SP, O'Boyle E, Morgello S (1993) Tissue distribution of the
358 human GLUT3 glucose transporter. Endocrinology 132: 2538-2543

359

Hahn D, Blaschitz A, Korgun ET, Lang I, Desoye G, Skofitsch G, Dohr G. From maternal glucose to fetal glycogen: expression of key regulators in the human placenta. Molecular human reproduction 7: 1173-1178, 2001.

Hediger MA, Rhoads DB. Molecular physiology of sodium- glucose cotransporters. *Physiol Rev* 1994; 74: 993-1026.

Horgun ET, Demir R, Hammer A, Dohr G, Scofitsch G, Hahn T (2001) Glucose transporter expression in rat embryo and uterus during decidualization, implantation, and early postimplantation. Biology of reproduction 65: 1364-1370

Ian A. Simpson, Donard Dwyer, Daniela Malide, Kelle H. Moley, Alexander Travis, and Susan J. Vannucci (2008) The facilitative glucose transporter GLUT3: 20 years of distinction. Am J Physiol Endocrinol Metab 295: E242-E253.

Illsey NP (2000) Glucose transporters in the human placenta. Placenta 21: 14-22.

Illsley NP, Sellers MC, Wright RL (1998) Glycaemic regulation of glucose transportes expression and activity in the human placenta. Placenta 19: 517-524.

Joost HG and Thorens B (2001) The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Molecular Membrane Biology 18, 247–256.

384 Lee WS, Kanai Y, Wells RG, Hediger MA (1994) The high affinity Na^+/glucose
385 cotransporter: re-evaluation of function and distribution of expression. J Biol Chem
386 269: 12032-12039
387
388 Limesand SW, Regnault TRH, Hay WW (2004) Characterization of glucose transporter
389 8 (GLUT8) in the ovine placenta of normal and growth restricted fetuses. Placenta 26:
390 70-7
391
392 Kim ST and Moley KH (2009) Regulation of facilitative glucose transporters and
393 AKT/MAPK/PRKAA signaling via estradiol and progesterone in the mouse uterine
394 epithelium. **Biol. Reprod** 81(1):188-98.
395
396 Knospe, C. (2002) Periods and stages of the prenatal development in the domestic cat.
397 Anat. Histol. Embryol. **31**, 37-51
398
399 Kuo Y, McQueen A, Chen TC, Wang JC (2015) Regulation of glucose homeostasis by
400 glucocorticoids. Adv Exp Med Biol 872: 99-126
401
402 Mueckler M, Caruso C, Baldwin SA, Panico M, Blench I, Morris HR, Allard WJ,
403 Lienhard GE, Lodish HF (1985) Sequence and structure of a human glucose transporter.
404 Science 229: 941-5
405
406 Nehlig A. Respective roles of glucose and ketone bodies as substrates for cerebral
407 energy metabolism in the suckling rat. Dev Neurosci 18: 426-433, 1996.
408

409 Ogura K, Sakata M, Okamoto Y, Yasui Y, Tadokoro C, Yoshimoto Y, Yamaguchi M,
410 Kurachi H, Maeda T, Murata Y (2000) 8-Bromo-cycli-cAMP stimulates glucose
411 transporter-1 expression in a human choriocarcinoma cell line. J Endocrinol 164: 171-
412 178
413
414 Pantaleon M, Harvey MB, Pascoe WS, James DE, Kaye PL. (1997) Glucose transporter
415 GLUT3: ontogeny, targeting, and role in the mouse blastocyst. Proc Natl Acad Sci USA
416 94: 3795-3800
417
418 Sabino-Silva R, Mori RC, David-Silva A, Okamoto MM, Freitas HS, Machado UF
419 (2010) The Na^+/glucose cotransporters: from genes to therapy. Braz J Med Biol Res 43:
420 1019-26
421
422 Sancho S[1], Casas I, Ekwall H, Saravia F, Rodriguez-Martinez H, Rodriguez-Gil
423 JE, Flores E, Pinart E, Briz M, Garcia-Gil N, Bassols J, Pruneda A, Bussalleu E,Yeste
424 M, Bonet S. 2007. Effects of cryopreservation on semen quality and the expression
425 of sperm membrane hexose transporters in the spermatozoa of Iberian pigs.
426 Reproduction 134(1):111-21.
427
428 Shepard PR, Gould GW, Colville CA, McCoid SC, Gibbs EM, Kahn BB (1992)
429 Distribution of GLUT3 glucose transporter in human tissues. Biochem Biophys Res
430 Commun 188: 149-154.
431

Shin B, Fujikura K, Suzuki T, Tanaka S, Tanaka K (1997) Glucose transporter-3 in the rat placental barrier: a possible machinery for the transplacental transfer of glucose. Endocrinology 138: 3997-4004

Sirois J and Doré M (1997) The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process. Endocrinology 138:4427–4434

Simpson IA, Dwyer D, Malide D, Moley KH, Travis A, Vannucci SJ (2008) The facilitative glucose transporter GLUT3: 20 years of distinction. AM J Physiol Endocrinol Metab 295: E242-53

Rigau T, Rivera M, M. J. Palomo MJ, Fernández-Novell JM, Mogas T, Ballester J, Peña A, Otaegui PJ, Guinovart JJ, Rodríguez-Gil JE (2002). Differential effects of glucose and fructose on hexose metabolism in dog spermatozoa. Reproduction 123: 579–591

Von Wolff M, Ursel S, Hahn U, Steldinger R, Strowitzki T 2003 Glucose transporter proteins (GLUT) in human endometrium: ex- pression, regulation, and function throughout the menstrual cycle and in early pregnancy. J Clin Endocrinol Metab 88:3885–3892

Wood IS, Trayhurn P. (2003) Glucose transporters (GLUT and SGLT): Expanded families of sugar transport proteins.

Wooding FBP, Dantzer VB, Klisch K, Jones CJ, Forhead AJ (2007) Glucose transporter 1 localisation throughout pregnancy in the carnivore placenta: Light and electron microscope studies. Placenta 28:453-464.

Wooding FBP, Fowden AL, Bell AW, Ehrhardt RA, Limesand SW, Hay WW (2005) Localisation of glucose transport in the ruminant placenta: implications for sequential use of transporter isoforms. Placenta 26:626-40

Wooding FBP, Morgan G, Fowden AL, Allen RW (2000) Separate sites and mechanisms for placental transport of calcium, iron and glucose in the equine placenta. Placenta 21: 635-45

Yamaguchi M, Sakata M, Ogura K, Miyake A. 1996 Gestational changes of glucose transporter gene expression in the mouse placenta and decidua. J Endocrinol Invest. 19(8):567-9.

Fig. 1. Western blotting expression for A) GLUT1 and B) GLUT3. Radiograph films showing a 55-60 kDa bands compatible with GLUTs. Note that the expression is present in all the evaluated groups. C+: Positive control; LP: non-pregnant queens with low levels of serum progesterone; HP: non-pregnant queens with high levels of serum progesterone; UT: uterus; TZ: transference zone; D30: 30 days of pregnancy; D40: 40 days of pregnancy; D50: 50 days of pregnancy; D60: 60 days of pregnancy. No statistically significant difference among the different groups was observed.

Fig 2. Immunohistochemistry images for GLUT1 expression. Cellular nuclei are blue-stained with DAPI dye, while GLUT1 is red-stained. **A:** Negative control in a kidney sample; **B:** Positive control in a rat kidney sample. Note the red mark for GLUT1 around the collecting ducts membrane of the kidney (white arrow); **C:** Uterine sample from a non-pregnant queen with low serum levels of progesterone. Note the red mark in the cellular membrane of the epithelial cells from the glandular epithelia (white arrows) and red mark in myometrial cells (white asterisk); **D:** Uterine sample from a non-pregnant queen with high serum levels of progesterone. Note the red mark in the cytoplasm of the cells from the glandular epithelia (white arrows) and the red mark in the myometrium (white asterisk); **E:** Uterine sample from D30 pregnancy. Note the red mark in the cytoplasm of the cells from the glandular epithelia (white arrow) and the red mark in the myometrium (white asterisk); **F:** Uterine sample from D40 pregnancy. Note the red mark in the cells from the endometrial glands (white arrow) and the red mark in the myometrium (white asterisk); **G:** Uterine sample from D50 pregnancy. Note the red mark in the cells from the endometrial glands and luminal epithelia (white arrow) and the red mark in the myometrium (white asterisk); **H:** Uterine sample from D60 pregnancy. Note the red mark in the cells from the endometrial glands and luminal

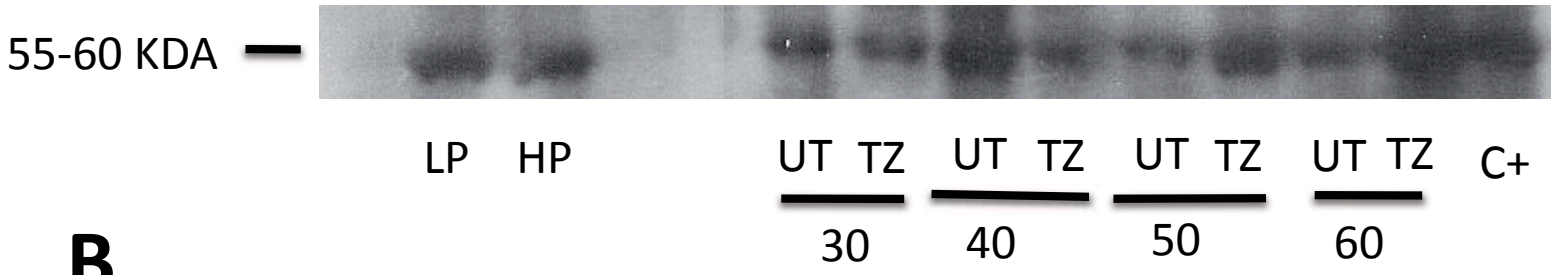
epithelia (white arrow) and the red mark in the myometrium (white asterisk); **I:** Sample from the transference zone (TZ) in a D30 pregnancy. Note the red mark in the apical membrane from the chorionic cells; **J:** Sample form the TZ in a D40 pregnancy. Note the red mark in the apical membrane from the chorionic cells; **K:** Sample from the TZ in a D50 pregnancy. Note the red mark in the apical membrane from the chorionic cells; **L:** Sample from the TZ in a D60 pregnancy. Note the red mark in the apical membrane from the chorionic cells.

Fig 2. Immunohistochemistry images for GLUT3 expression. Cellular nuclei are blue-stained with DAPI dye, while GLUT3 is green-stained. **A:** Negative control in a chorionic sample; **B:** Positive control in a rat small intestine sample. Note the green mark for GLUT3 around the intestinal villy (white arrow); **C:** Uterine sample from a non-pregnant queen with low serum levels of progesterone. Note the green mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **D:** Uterine sample from a non-pregnant queen with high serum levels of progesterone. Note the green mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **E:** Uterine sample from D30 pregnancy. Note the green mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **F:** Uterine sample from D40 pregnancy. Note the green mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **G:** Uterine sample from D50 pregnancy. Note the green mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **H:** Uterine sample from D60 pregnancy. Note the green mark in the cellular membrane of the epithelial cells from the glandular and luminal epithelia (white arrows); **I:** Sample from the transference zone (TZ) in a D30 pregnancy. Note the green mark in the chorionic cells; **J:** Sample form the TZ in a D40

pregnancy. Note the green mark in the chorionic cells; **K:** Sample from the TZ in a D50 pregnancy. Note the green mark in the chorionic cells; **L:** Sample from the TZ in a D60 pregnancy. Note the green mark in the chorionic cells.

Table 1. Relative GLUT1 and 3 abundance calculated by means of the Multi Gauge v3.0 software system. Statistically significant differences at different sexual and pregnancy stages for each AQP are indicated with different superscripts.

A


55-60 KDA
LP
HP
UT
TZ
UT
TZ
UT
TZ
UT
TZ
C+
30
40
50
60

B

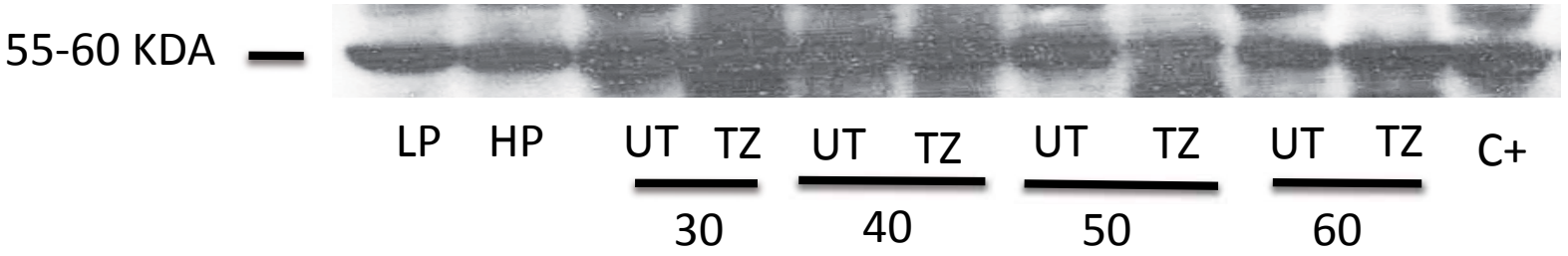
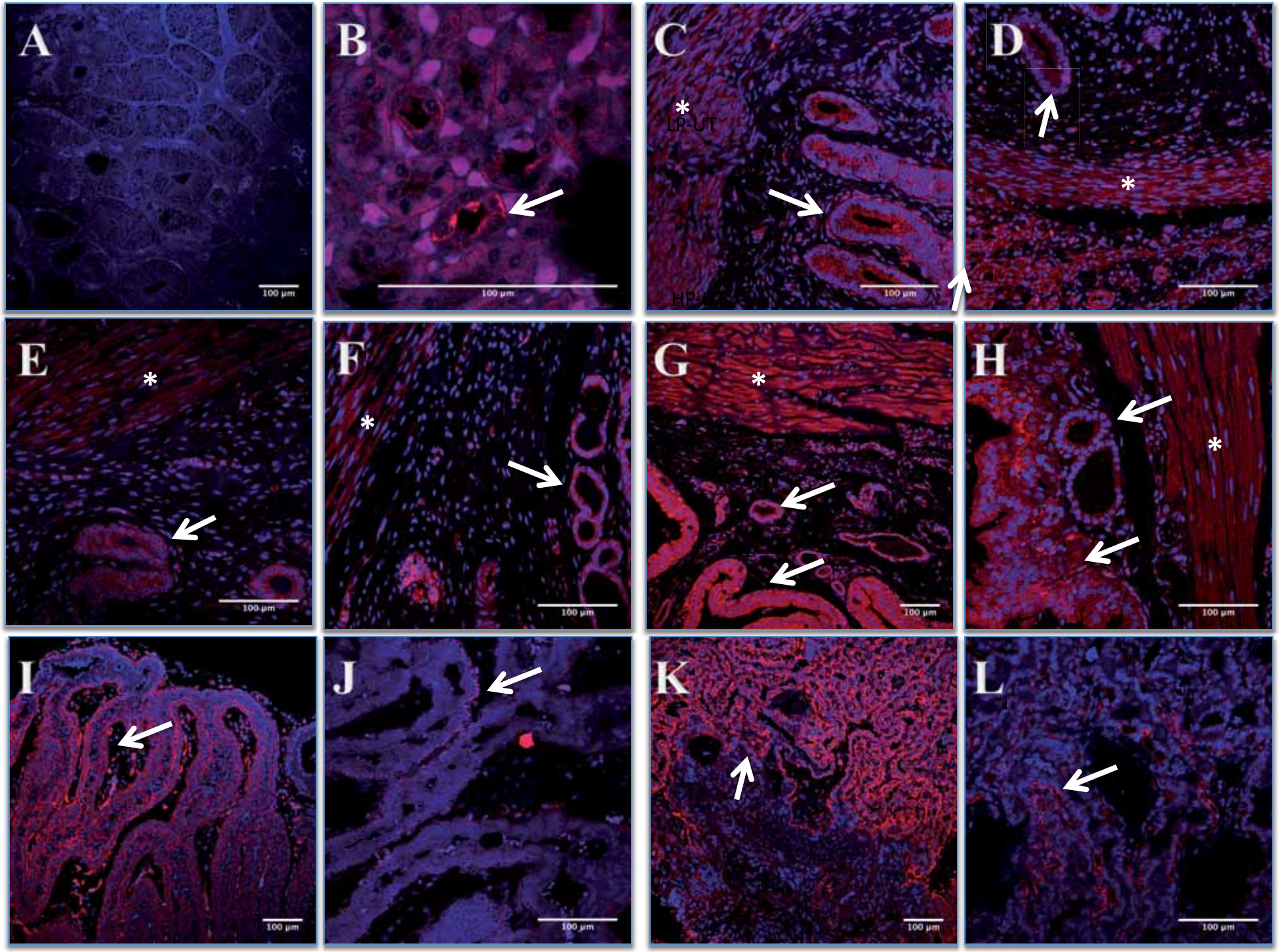
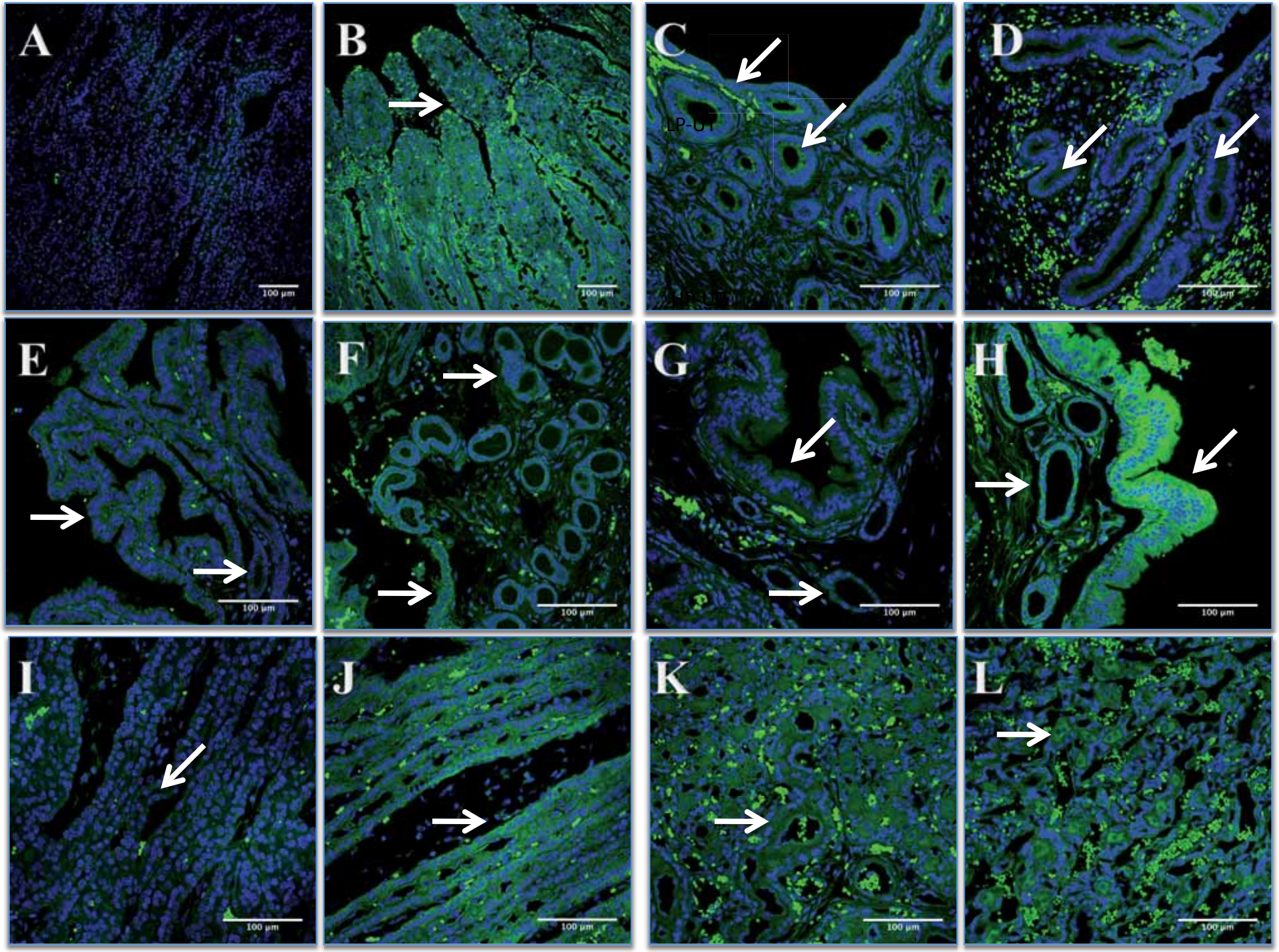

55-60 KDA
LP
HP
UT
TZ
UT
TZ
UT
TZ
UT
TZ
C+
30
40
50
60

Fig. 1


A
B
C
*
LP-UT
D
*
E
*
F
*
G
*
H
*
I
J
K
L
100 µm


A
B
C
D
E
F
G
H
I
J
K
L
100 µm

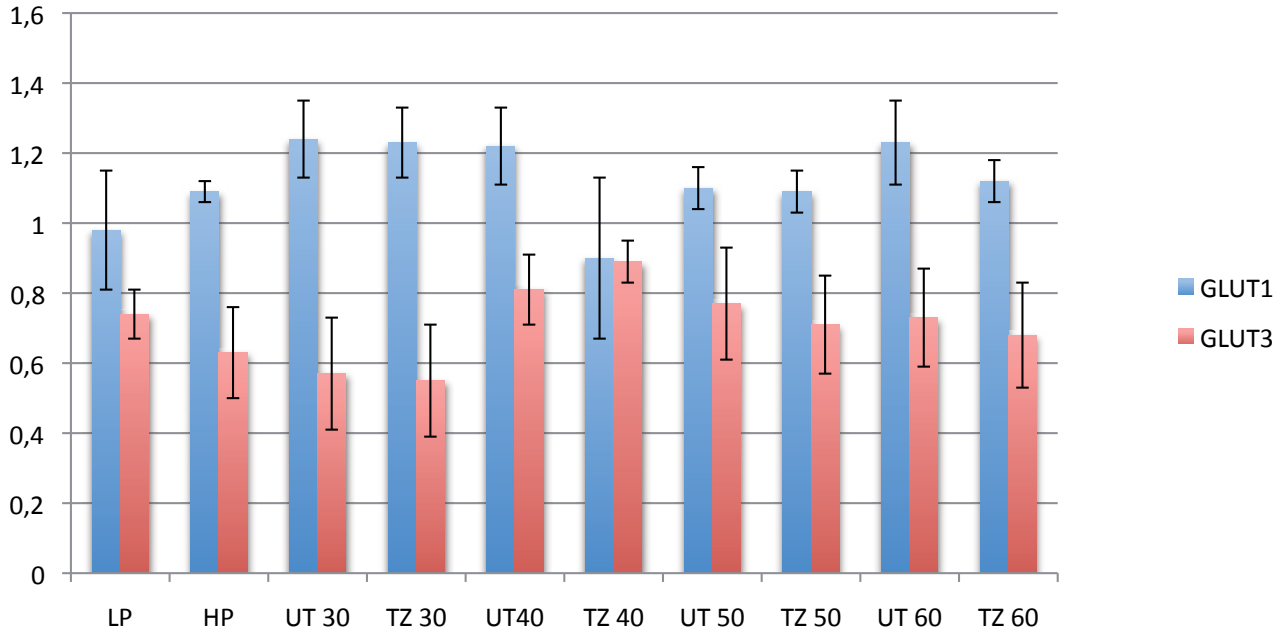

1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
LP
HP
UT 30
TZ 30
UT40
TZ 40
UT 50
TZ 50
UT 60
TZ 60
GLUT1
GLUT3

Table 1

General discussion

Several studies have been focused on the importance of water movement across the female reproductive tract. This event is not only important at the ovulation timing for the creation of the uterine edema, but also for oocyte transport or amniotic fluid balance during pregnancy (Sha *et al.*, 2011). The regulation of water across the uterus and the placenta is a poorly understood mechanism, but this phenomenon seems to involve aquaporins (AQPs) as major participants.

The results of the present study reveal that AQP1, 2, 3 and 8 are present in queen uterus. Some studies have previously described that the expression of AQPs in the uterus is regulated by ovarian steroid hormones such as E2 and P4, suggesting that these AQPs play important roles in hormone-mediated water and other solute transport in uterine imbibition (He *et al.*, 2006, Aralla *et al.*, 2009, Skowronski *et al.*, 2010). In our studies, changes in cellular location of AQP2 and 8 related to serum progesterone concentration were observed. In non-pregnant queens with low levels of progesterone (progesterone $\leq$ 1 ng/mL), AQP2 and 8 were distributed in the plasma membrane of both glandular and laminal epithelia of the uterus. In contrast, in non-pregnant queens with high levels of progesterone (progesterone $\geq$ 2 ng/mL) and pregnant queens, AQP2 and 8 were distributed in both plasma membrane and along the entire cytoplasm of both glandular and laminal epithelia, suggesting that serum progesterone concentration induces modifications of the intracellular distribution of both AQPs. Nevertheless, not enough data is available concerning to the changes in cellular distribution of these transporters related to ovarian steroid hormones and further research is needed to establish if progesterone really controls APQ2 and 8 function in the uterus.

On the other hand, the abundance of AQPs did not changed along the pregnancy period or the different sexual phases, nor AQP2 and 8 neither AQP1 and 3. These results are in disagreement with some previous studies, which have observed changes in the expression of APQs1, 2, 5 and 9 related with serum progesterone concentration. It is important to highlight that fact that, due to the intimate endotheliochorial placenta of the queen, samples from pregnant queens for immunoblotting analyses were difficult to split into chorion and endometrium and were probably analyzed as a unique unit. Thus, a possible change in the relative abundance of AQPs could have been masked. Actually, if we take into account the changes observed in the distribution of AQP2 and 8 related with levels of serum progesterone, that would be a more than feasible explanation.

Surprisingly, although there was no change in the expression of AQPs during the different sexual and pregnancy stages, AQP1 negatively correlated with serum progesterone levels, suggesting that AQP1 is maybe down-regulated by progesterone.
In addition to the endometrium, and in agreement with other studies in human and rat (Mobasheri *et al.*, 2005; Lindsay *et al.*, 2006), AQP 1, 2 and 3 were expressed at the myometrial cells from queens, suggesting that the presence of water transporters in the myometrium may regulate the water flux from the myometrial cells to the endometrial surface for the preparation of the endometrium for implantation or other modifications during pregnancy (Richard *et al.*, 2003). Nevertheless, the expression of these aquaporins has not been yet related to the muscle contraction or metabolism of the myometrium, being unclear its function.
Regarding to the placenta, many authors have hypothesized that AQPs may play a role in the amniotic fluid reabsorption (Liu *et al.*, 2008, Sha *et al.*, 2011, Aralla *et al.*, 2012).

Many authors have described the presence of AQP1 in the chorionic vascular endothelium of different species such as human, mouse and bitch (Mann *et al.*, 2002, Zhu *et al.*, 2009, Aralla *et al.*, 2012). However, our results are in disagreement with these previous studies. The chorionic surface of the queen placenta did not express AQP1, suggesting that AQP1 may not be involved in the water flow to the embryo during the queen pregnancy. This result, combined with the negative correlation of AQP1 and progesterone in the endometrium described above, would support the hypothesis that AQP1 is maybe down-regulated during phases with high levels of progesterone.
On the other side, AQP2, 3 and 8 showed were expressed in the chorionic layer. This fact suggests that AQP2, 3 and 8 may take part in the water supply to the embryo and play a role in water disposal. In addition to water, AQP8 has been described to transport urea. In the present study, AQP8 showed a high expression in the cells from the chorionic labyrinth, which is in accordance to Aralla *et al.*, (2009) and Zhu *et al.*, (2010). As the urea produced by the fetus has to be removed from the fetal circulation to the maternal circulation, it can be hypothesized that AQP8 may be involved in the urea transport across the fetal membranes. Regarding to AQP3, this AQP showed a higher immunoreactivity at the cytotrophoblasts than in the syncytiotrophoblasts, suggesting a

water flow across the chorionic surface and the maternal endometrium for the fetal development.

Finally, in order to study the activity of AQP2 during pregnancy, the phosphorylation of Tyr-residues of this protein was evaluated. Several authors (Katsura *et al.,* 1997; He *et al.,* 2006; Moeller *et al.,* 2009) have described that the phosphorylation of Tyr-residues modulates the endocytosis and exocytosis of AQP2 in cells. In our study, variations of TyrPhos of AQP2 were not related to changes of AQP2 progesterone-related location modifications. This fact suggests that progesterone is the responsible of cellular distribution of AQP2 and not its phosphorylation in Tyr-residues.

It is easy to think that various isoforms of AQPs presents in different cell compartments can modulate water movement. According to our results, every AQP has a particular location in both queen uterus and placental transfer zone, being located in endothelia, myometrial cells, epithelia and chorionic surface. In addition, AQPs can be located in the plasma membrane or in the whole cytoplasm. When compared to other species, these locations are quite different,. AQP1 in queens was not present in vascular endothelia as it happens in bitch, mice or human (Mann *et al.,* 2002, Zhu *et al.,* 2009, Aralla *et al.,* 2012).. Moreover, AQP2 and 8 showed a change of location from the plasma membrane to the whole cytoplasm according to progesterone concentration. As it has been aftermentioned, queen reproductive tract and placenta have certain differences in terms of anatomy and physiology. This fact can suggest different sites of action, metabolic action and role of these proteins in queen reproductive tract.

Regarding to glucose transporters, both GLUT1 and GLUT3 were expressed in queen uterus and placental transfer zone, which is in accordance with previous studies that have observed the presence of glucose transporters in rat and human (Hahn *et al.,* 1995; Hahn *et al.,* 2000). Anyway, there are few data about glucose transporters in the reproductive system and its role in these tissues is not fully yet understood.

Some authors have described a correlation between the expression of GLUT1 in the female reproductive tract of mice and human with serum levels of progesterone (Yamaguchi *et al.,* 1996; Frolova *et al.,* 2009). However, our results are in disagreement. In queens, the expression of GLUT1 in both uterus and placental transference zone is not correlated with progesterone levels. On the other hand, when GLUT3 expression was evaluated comparing to serum levels of progesterone, non-

pregnant queens with low levels of serum progesterone expressed more GLUT3 than both pregnant and non-pregnant queens with high levels of progesterone, although no statistically significant correlation was observed as it has been previously stated. In addition, queens with low levels of serum progesterone express more GLUT3 than queens with high levels of serum progesterone. These results can suggest that at time of implantation, other transporters in addition to GLUT3 may be involved in the preparation for embryo implantation, where glucose requirements may be increased.

Simpson *et al.* (2008) described that GLUT1 was the glucose transporter most expressed in the endometrium. However, in the present study, both GLUT1 and showed the same relative abundance in the different sexual and pregnancy phases.

On the other hand, it is well known that GLUT3 has a high affinity for glucose. However, GLUT3 is able to transport other hexoses such as mannose, xylose and sacarose. This fact suggests that not only glucose uptake is necessary in the endometrium, but also other kind of glucose.

Several authors (Esterman *et al.*, 1997, Hahn *et al.*, 2001, Simpson *et al.*, 2008) described that GLUT3 is expressed in mouse and human placenta only during the first trimester of pregnancy. However, GLUT3 was expressed in the chorionic layer of all the pregnancy stages in queens. This controversy may suggest that differences between species or types of placenta could determine the mechanism responsible for glucose transport from the dam to the fetus.

Regarding to GLUTs location, the present study describes the presence of GLUT1 in the endothelial cells of endometrial vessels, being in disagreement with what other authors have published in queens (Wooding *et al.*, 2007). These differences between the studies could be to the technique or the use of newest antibodies with higher sensitivity to GLUT1 protein. Moreover, the innovation of techniques in both immunoblotting and immunohistochemistry may be determinant in the detection of this protein.

On the other hand, as Brown *et al.*, (2011) described in human placenta, GLUT3 is present in the endothelia of the chorionic membranes, but not in the maternal vessels of the endometrium. This result suggests that GLUT3 may be a critical mediator of glucose uptake from the maternal circulation to the trophoblastic cells and the energetically demanding fetus.

Nevertheless, other authors described that GLUT3 was not present from second trimester of pregnancy on (Esterman *et al.*, 1997, Hahn *et al.*, 2001, Simpson *et al.*, 2008). However, queen placentation totally differs from mouse and human placentation. These characteristics may be important for supporting our results, suggesting that in the endotheliochorial placentas, transport of solutes from the mother circulation should be done until delivery.

Conclusions

The results of the present thesis lead to the next conclusions:

1. AQPs 1, 2, 3 and 8 are present in the cells from both luminal and glandular epithelia of the queen endometrium, including the placental transference zone.
2. AQPs 1, 3 and 8 did not show any significant difference in their expression in the cells from luminal and glandular epithelia from the queen endometrium in the different sexual and gestational stages.
3. Uterine samples from non-ovulated queens showed significant higher AQP2 intensity than uterine samples form 50 days of pregnancy and placental transference zone samples of 30 and 50 days of pregnancy.
4. AQP1 is not present in the chorionic layers of queen placenta.
5. AQP 2, 3 and 8 are present in the chorionic layer of the queen placenta along the pregnancy.
6. AQPs 1, 3 and 8 did not show any significant difference in their expression in the placental transference zone along the pregnancy.
7. AQP2 activity in the feline endometrium and placenta is not regulated by the Tyrosine phosphorylation of this AQP.
8. AQP1 is negatively correlated with serum progesterone levels while AQP3 is positively correlated. AQP2 and AQP8 did not show any correlation with serum progesterone levels.
9. Serum progesterone levels induce changes in the cellular location of AQP2 and AQP8. When progesterone concentration is less than 1 ng/ml, these AQPs are located only in the cell membrane. However, when progesterone levels are greater than 2 ng/ml, these AQPs are located in both cell membrane and cytoplasm.
10. AQP8 expression in the chorionic layer suggests that this AQP is involved in the metabolism or elimination of urea from the feline fetus.
11. GLUTs 1 and 3 are present in the cells from both luminal and glandular epithelia of the queen endometrium.
12. GLUTs 1 and 3 are present in the chorionic layer of every gestational stage evaluated.
13. GLUT1 and 3 did not show any significant difference in their expression in the cells from luminal and glandular epithelia from the queen endometrium.

14. GLUT1 and 3 did not show any significant difference in their expression, placental transference zone at gestational stages.
15. GLUT3 is negatively correlated with serum progesterone levels while GLUT1 did not show any correlation.

References

A

Abrami L, Capurro C, Ibarra C, Parisi M, Buhler JM, Ripoche P (1995) Distribution of mRNA encoding the FA-CHIP water channel in amphibian tissues: effects of SALT adaptation. J Membr Biol 143, 199-205

Agre P, Saboori AM, Asimos A, Smith BL (1987) Purification and partial characterization of the Mr 30000 integral membrane protein associated with the erythrocyte Rh (D) antigen. J Biol Chem. 262: 17497-17503.

Agre P, Preston GM, Smith BL, Jung JS, Raina S, Moon C, Guggino WB, Nielsen S (1993) Aquaporin CHIP: the archetypal molecular water channel. Am J Physiol. 265: F463- F476

Agre P, King LS, Yasui M, Guggino WB, Ottersen OP, Fujiyoshi Y, Engel A, Nielsen S (2002) Aquaporin water channel-from atomic structure to clinical medicine. J Physiol. 542:3–16

Agre P. (2004) Nobel lecture. Aquaporin water channels. Biosci Rep. 24:127-63

Anderson J, Brown N, Mahendroo MS, Reese J (2006) Utilization of different aquaporin water channels in the mouse cervix during pregnancy and parturition and in models of preterm and delayed cervical ripening. Endocrinology 147(1): 130-40.

Angulo C, Rauch MC, Droppelmann A, Reyes AM, Slebe JC, Delgado-Lopez F, Guaiquil VH, Vera JC, Concha II (1998) Hexose transporters expresión and function in mammalian spermatozoa: Cellular localization and transporto f hexoses and vitamina C. Biochem Cell Biol 71: 189-203

Aralla M, Borromeo V, Groppetti D, Secchi C, Cremonesi F, Arrighi S (2009) A collaboration of aquaporins handles water transport in relation to the estrous cycle in the bitch uterus. Theriogenology. 72: 310-321

Aralla M, Mobasheri A, Groppetti D, Cremonesi F, Arrighi S (2012) Expression of aquaporin water channels in canine fetal adnexa in respect to the regulation of amniotic fluid production and absorption. Placenta. 33: 502-510.

Arrigi S, Ventriglia G, Aralla M, Zizza S, Di Summa A, Desantis S (2010) Absorptive activities of the efferent ducts evaluated by the immunolocalization of aquaporin water channels and lectin histochemistry in adult cats. Histol Histopathol 25: 433-444

Arrighi S & Aralla M (2014) Immunolocalization of aquaporin water channels in the domestic cat male genital tract. Reproduction in domestic animals 2014; 49: 17-26.

B

Barcroft LC, Offenberg H, Thomsen P, Watson AJ (2003) Aquaporin proteins in murine thophoectoderm mediate transepithelial water movements Turing cavitation. Dev Biol 256, 342-354

Banerjee A, Anuradha, Mukherjee K, Krishna A (2014) Testicular glucose and its transporter GLUT8 as a marker of age-depending variation and its role in steroidogenesis in mice. J Exp Zool A Ecol Genet Physol 321(9): 490 - 502

Barros LF, Baldwin A, Jarvis SM, Cowen T, Thrasivoulou C, Beaumont N, Yudilevich DL (1992) In human placenta the erythroid glucose transporter Glut1 is abundant in the brush-border and basal membranas of the trophoblast but the insulina-dependent isoforma Glut4 is absent-neither transporte ris detectable in endotelial cells. J physiol London. 446: 345.

Beall MH, Wang S, Yang B, Chaudhri N, Amidi F, Ross MG (2007) Placental and membrane aquaporin water channels: Correlation with amniotic fluid volumen and composition. Placenta. 28: 421-428.

Beitz E, Kumagami H, Krippeit-Drews P, Ruppersberg JP, Schultz JE (1999) Expression pattern of aquaporin water channles in the inner ear of the rat. The

molecular basis for a water regulation system in the endolymphatic sac. Hear Res. 132: 76-84.

Bell GI, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, Fukumoto H, Seine S (1990) Molecular biology of mammalian glucose transporters. *Diabetes Care*. 13: 198-208.

Braés MC, Morales B, Ríos M, Villalón MJ (2005) Regulation of the inmunoexpression of aquaporin 9 by ovarian hormones in the rat oviductal epithelium. AM J Physiol Cell Physiol. 288: C1048-57.

Brandan HH & Hermo LS (2002) Expression and regulation of aquaporins 1, 8 and 9 in the testis, efferent ducts and epididymis of adult rats and during postnatal development. J Androl 20, 158-176.

Brown D, Verbavatz JM, Valenti G, Lui B, Sabolic I (1993) Localization of the CHIP28 water channel in reabsorptive segments of the rat male reproductive tract. Eur J Cell Biol 61,264–273.

Bryant NJ, Govers R & James DE (2002) Regulated transport of the glucose transporter GLUT4. Nature Reviews Molecular Cell Biology 3, 267–277.

Bucci D, Isani G, Spinaci M, Tamanini C, Mari G, Zambelli D, Galeati G (2010) Comparative immunolocalization of GLUTs 1, 2, 3 and 5 in boar, stallion and dog spermatozoa. Reprod, Domest Anim 45(2): 315-22

Burant CF, Takeda J, Brot-Laroche E, Bell GI, Davidson NO (1992) Fructose transporter in human spermatozoa and small intestine is GLUT5. J Biol Chem 267(21): 14523-14526

Burant CF & Davidson NO (1994) GLUT3 glucose transporter isoform in rat testis: localization, effect of diabetes mellitus, and comparison to human testis.

C

Calamita G, Mazzone A, Cho YS, Valenti G, Svelto M (2001a) Possible involvement of aquaporin-7 and -8 in rat testis development and spermatogenesis. Biochem Biophys Res Commun 288, 619-625.

Calamita G, Mazzone A, Cho YS, Valenti G, Svelto M (2001b) Expression and localization of the aquaporin-8 water channel in rat testis. Biol Reprod 64, 1660-1666

Carayannopoulos MO, Chi MM, Cui Y, Pingterhaus JM, McKnight RA, Mueckler M, Devaskar SU, Moley KH (2000) GLUT8 is a glucose transporter responsable for insulin-stimulated glucose uptake in the blastocyst. PNAS 977: 313–7318

Carayannopoulos MO, Schlein A, Wyman A, Chi M, Keembiyehetty C, Moley KH (2004) GLUT9 is differentially expressed and targeted in the preimplantation embryo. Endocrinology 145(3): 1435-1443.

Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yo-shinaga K (2000) Embryo implantation. Dev Biol 223:217–237.

Clemenston CA, Verma UL, De Carlo SJ (1977) Secretion and reabsorption of uterine luminal fluid in rats. J Reprod Fertil 49, 183-187

Clulow J, Jones RC, Hansen LA, Mann SY (1998) Fluid and electrolyte reabsorption in the ductuli efferentes testis. J Reprod Fertil Suppl 53, 1-14

Cullinan-Bove K & Koos RD (1993) Vascular endothelial growth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. Endocrinology 133: 829-837

D

Da Silva N, Silberstein C, Beaulieu V, Pietrement C, Van Hoek AN, Brown D, Breton S (2006) Postnatal expresión of aquaporins in epithelial cells of the rat epididymis. Biol Reprod 74, 427-438.

Damiano AE (2011) Review: Water channel proteins in the human placenta and fetal membranes. Placenta. 25:S207-S211.

Dawson PA, Mychaleckyj JC, Fossey SC, Mihic SJ, Craddock AL, Bowden DW (2001) Sequence and functional analysis of GLUT10: a glucose transporter in the Type 2 diabetes-linked region of chromosome 20 12-13. Mol Genet Metab 74: 186-1999.

De Falco M, Cobellis L, Torella M, Acone G, Varano L, Sellitti A, A Ragucci, Coppola G, R Cassandro (2007) Downregulation of aquaporin 4 in human placenta throughout pregnancy. *In Vivo* 21, 813-817

Deen PM & Knoers NV (1998) Vasopressin type-2 receptor and aquaporin-2 water channel mutants in nephrogenic diabetes insipidus. Am J Med Sci. 316: 300-309.

Denker BM., Smith BL., Kuhajda FP, Agre, P (1988) Identification, purification, and partial characterization of a novel Mr 28,000 integral membrane protein from erythrocytes and renal tubules. *Journal of Biological Chemistry* 263, 15634–15642.

Desoye G & Shafrir E (1994) Placental metabolism and its regulation in health and diabetes. Mol Aspects Med 15: 505 - 682

Desoye G, Gauster M, Wadsack C (2011) Placental transport in pregnancy pathologies. Am J Clin Nutr 94(6): 1896S – 1902S

Doege H, Bocianski A, Scheepers A, Axer H, Eckel J, Joost HG, Schurmann A (2001) Characterization of human glucose transporter (GLUT) 11 (encoded by SLC2A11), a

novel sugar-transport facilitator specifically expressed in heart and skeletal muscle. Biochemical Journal 359, 443–449.

Doege H, Schürmann A, Bahrenberg G, Brauers A, Joost HG (2000b) GLUT8, a novel member of the sugar transport facilitador family with glucose transport activity. J Biol Chem 275(21): 16275-16280.

E

Edashige K, Sakamoto M, Kasai M (2000) Expression of mRNAs of the aquaporin family in Mouse oocytes and embryos. Cryobiology 40, 171-175.

Edashige K, Yamaji Y, Kleinhans FW, Kasai M (2003) Artificial expresión of aquaporin 3 improves the survival of Mouse oocytes alter cryopreservation. Biol Reprod 68, 87-94.

Edwards D, Jones CJ, Sibley CP, Nelson DM (1993) Paracellular permeability pathways in the human placenta: a quantitative and morphological study of maternal transfer of horseradish peroxidase. Placenta. 14(1): 63-73.

Enders AC, Carter AM (2006) Comparative placentation: some interesting modifications for histotrophic nutritionda review. Placenta. 27 Suppl A:S11–6.

F

Fatin, R., Orsi, A.M., Justulin, L.A., Leme, C.C., Felisbino, S.L. (2008) Immunolocalization of aquaporins 1, 2 and 7 in rete restis, efferent ducts, epididymis and vas deferens of adult dog. Cell Tissue Res. 332, 329-335.

Finlay TH., Katz J., Kirsch L., Levitz M., Nathoo SA., Seiler S. (1983) Estrogen-stimulated uptake of plasminogen by the mouse uterus. Endocrinology 112, 856-861

Floehner SC, Davies A, Baldwin SA, Lienhard GE (1988) The blood nerve barrier is rich in glucose trasporter. J Neurocytol. 17: 173-178.

Frigeri A, Nicchia GP, Verbavatz JM, Valenti G, Svelto M (1998) Expression of aquaporin-4 in Fast-twitch fibers of mammalian skeletal muscle. J Clin Invest. 102: 695-703

Frolova A, Flessner L, Chi M, Kim ST, Foyouzi-Yousefi N, Moley KH (2009) Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells. Endocrinology 150:1512–1520

Frolova AI & Moley KH (2011) Quantitative analysis of glucose transporter mRNAs in endometrial stromal cells reveals critical role of GLUT1 in uterine receptivity.Endocrinology. 152: 2123–2128

G

Galantino-Homer HL, Florman HM, Storey BT, Dobrinski I, Kopf GS. (2004) Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalinization on capacitation-associated proteína tyrosine phosphorylation. Mol Reprod Dev 67: 487-500.

Gannon BJ, Warnes GM, Carati CJ, Verco CJ (2000) Aquaporin 1 expression in visceral smooth muscle cells of female reproductive tract. J Smooth Muscle Res 36, 155-167

Gardner DK, Leese HJ (1988) The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantational embryos. Development 104: 423-429

Gomes D., Agasse A., Thiébaud P., Delrot S., Gerós H., Chaumont F (2009) Aquaporins are multifunctional water and solute transporters highly divergent in living organisms. Biochimica et Biophysica Acta. 1213-1228

Gude N, Stevenson J, Rogers S, Best JD, Kalionis B, Huisman MA, Erwich JJ, Timmer A, King RG (2003). GLUT12 expression in human placenta in first trimestre and term. Placenta 24(5): 566-70

H

Haber RH, Wienstein SP, O'Boyle E, Morgello S (1993) Tissue distribution of the human GLUT 3 glucose transporter. Endocrinology 132-6: 2538-2543

Hahn D, Blaschitz A, Korgun ET, Lang I, Deyose G, Skofisch G, Dohr G (2001) From maternal glucose to fetal glycogen: expresión of key regulators in the human placenta. Mol Hum Reprod 7(12): 1173 - 1178

Hahn T, Hartmann M, Blaschitz A, Skofitsch G, Graf R, Dohr G, Desoye G (1995) Localisation of the high affinity facilitative glucose transporter protein GLUT 1 in the placenta of human, marmoset monkey (Callithrix jacchus) and rat at different developmental stages. Cell Tissue Res 280:49–57

Hahn T, Barth S, Weiss U, Mosgoeller W, Desoye G (1998) Sustained hyperglycemia in vitro down-regulates the GLUT1 glucose transport system of cultured human term placental trophoblast: a mechanism to protect fetal development? FASEB J**12**:1221–31

Hahn T, Hahn D, Blaschitz A, Desoye G, Korgun ET, Dohr G (2000) Hyperglycaemia-induced subcellular redistribution of GLUT1 glucose transporters in cultured human term placental trophoblast cells. Diabetologia **43**:173–80

Harik SI, Kalaria RN, Whitney PM, Andersson L, Lundhal P, Ledbetter SR, Perry G (1990) Glucose transporter are abundant in cells with occluding junctions at the eye blood barriers. Proc Natl Acad Sci USA. 87: 4261-4264.

He RH, Sheng JZ, Luo Q, Jin F, Wang B, Qian YL, Zhou CY, Sheng X, Huang HF (2006) Aquaporin-2 expression in human endometrium correlates with ovarian steroid hormones. Life Sci. 79: 423-429.

Hermo L, Krzeczunowicz D, Ruz R (2004) Cell specificity of aquaporins 0, 3 and 10 expressed in the testis, efferent ducts and apididymis of adult rats. J Androl 25, 494-505.

Hogan A, Heyner S, Charron MJ, Copeland NG, Gilbert DJ, Jenkins NA, Thorens B, Schultz GA (1991) Glucose transporter gene expresión in early Mouse embryos. Development 113: 363-372.

Hong SW, Chung HM, Lim JM, Ko JJ, Yoon TK, Yee B, Cha KY (1999) Improved human oocyte development alter vitrification: a comparison of thawing methods. Fertil Steril 72, 142-146.

Huang HF, He RH, Sun CC, Zhang Y, Meng QX, Ma YY (2006) Function of aquaporins in female and male reproductive Systems. Human Reproduction Update. 12: 785-795.

I

Ishibashi K, Kuwahara M, Gu Y, Kageyama Y, Tohsaka A, Suzuki F, Marumo F, Sasaki S (1997) Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. J Biol Chem . 272: 20782–6.

Ishibashi K, Kuwahara M, Gu Y, Kageyama Y, Tohsaka A, Suzuki F, Marumo F, Sasaki S (1997b) Cloning and functional expresion of a new water channel abundantly expressed in the testis permeable to water, glycerol and urea. J Biol Chem. 272: 20782-20786.

Ishibashi K, Kuwahara M, Gu Y, Kageyama Y, Tohsaka A, Suzuki F, Marumo F, Sasaki S (1997c) Cloning and functional expresión of a second new aquaporin abundantly expressed in testis.. Biochem Biophys Res Commun. 273: 714-718.

Ishibashi K (2009) New members of mammalian aquaporins: AQP10 - AQP12. Handb Exp Pharmacol. 190:251-62.

J

Jablonski EM, McConell NA, Hughes FM Jr, Huet-Hudson YM (2003) Estrogen regulation of aquaporins in the Mouse uterus: potencial roles in uterine water movement. Biol Reprod. 69: 1481-1487.

Johns A, Buchannan JD, Coons LW (1982) Effect of ovulation on the ionic and water content of rabbit oviduct. Biol Reprod 26, 367-377

Johnston SD, Root Kustritz MV, Olson PNS (2001).Canine and feline theriogenology. ed. Saunders, Philadelphia, PA.

Johnston H, Koukoulas I, Jeyaseelan K, Armugam A, Earnest L, Baird R, Dawson N, Ferraro T, Wintour EM (2000) Ontogeny of aquaporins 1 and 3 in ovine placenta and fetal membranes. Placenta 21:88-99.

Joost HG & Thorens B (2001) The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Molecular Membrane Biology 18, 247–256.

K

Kalaria RN, Gravina SA, Schmidley JW, Perry G, Harik SI (1988) The glucose transporter of the human brain and blood-brain-barrier. Ann Neu. 24: 757-64

Kayano T, Fukumoto H, Eddy RL, Fan YS, Byers MG, Shows TB, Bell GI (1988) Evidence for a family of human glucose transporter-like proteins: sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues. J Biol Chem 263: 15245-15248.

Kayano T, Burant CF, Fukumoto H, Gould GW, Fan YS, Eddy RL, Byers MG, Shows TB, Seino S, Bell GI (1990) Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoforms (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogenelike sequence (GLUT6). J Biol Chem 265: 13276-13282.

Kim ST & Moley KH (2007) The expresión of GLUT8, GLUT9a and GLUT9b in the Mouse testis and sperm. Reprod Sci 14(5): 445 - 55

Kim ST & Moley KH (2009) Regulation of facilitative glucose transporters and AKT/MAPK/PRKAA signaling via estradiol and progesterone in the mouse uterine epithelium. Biology of Reproduction 81: 188–198

King LS, Yasui M, Agre P (2000) Aquaporins in health and disease. Mol Med Today. 6: 60-65.

Klein C, Troedsson MHT, Rutllant J (2013) Expression of aquaporin water channels in equine endometrium is differentially regulated during the oestrus cycle and early pregnancy. Reproduction of Domestic Animals. 48: 529-537.

Kobayashi K & Yasui M (2010) Cellular and subcellular localization of aquaporins 1, 3, 8, and 9 in amniotic membranes during pregnancy in mice. *Cell Tissue Res* 342, 307-16.

Kohan K, Carvajal R, Gabler F, Vantman D, Romero C, Vega M (2010) Role of the transcriptional factors FOXO1 and PPARG on gene expression of SLC2A4 in endometrial tissue from women with polycystic ovary syndrome. Reproduction 140: 123–131

Korgun ET, Demir R, Hammer A, Dohr G, Desoye G, Skofitsch G, Hahn T (2001) Glucose transporter expression in rat embryo and uterus during decidualization, implantation, and early postimplantation. Biology of Reproduction **65**1364–1370

Korgun ET, Celik-Ozenci C, Seval Y, Desoye G & Demir R (2005) Do glucose transporters have other roles in addition to placental glucose transport during early pregnancy? Histochemistry and Cell Biology 123 621–629

Koyama N, Ishibashi K, Kuwahara M, Inase N, Ichioka M, Sasaki S, Marumo F (1998) Clonind and functional expresión of human aquaporin 8 cDNA and análisis of its gene. Genomics 54, 169-172

L

Leiser, R. (1979) Blastocystenimplantation bei der Hauskatze. Licht- und elektronenmikroskopische Untersuchung. Zbl. Vet. Med. C, Anat. Histol. Embryol. 8:79-96

Levine N and Marsh DJ (1971) Micropuncture Studies of the electrochemical aspects of fluid and electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. J Physiol 213, 557-570

Li X, Yu H, Koide SS (1994) The water channel gene in human uterus. Biochem Mol Biol Int 32:371–7.

Li X, Yu H, Koide SS (1997) Regulation of water channel gene (AQP-CHIP) expresion by estradiol and anordiol in rat uterus. Yao Xue Xue Bao. 32: 586-592.

Li Q, Manolescu A, Ritzel M, Yao S, Slugoski M, Young JD, Chen XZ, Cheeseman CI (2004) Cloning and functional characterization of the human GLUT7 isoform SCL2A7 from the small intestine. AM J. Physiol. Gastrointest. Liver Physiol. 287(1): G236-242

Lindsay LA & Murphy CR (2006) Redistribution of aquaporins 1 and 5 in the rat uterus is dependent on progesterone: a study with Light and electron microscopy. Reproduction. 131:369-378.

Lisinski I, Schürmann A, Joost HG, Cushman SW, Al-Hasani H (2001) Targeting of GLUT6 (formerly GLUT9) and GLUT8 in rat adipose cells. Biochem J 358: 517-522

M

Ma T, Yang B, Verkman AS (1997) Cloning of a novel water and urea-permeable aquaporin from mouse expressed strongly in colon, placenta, liver, and heart. *Biochem Biophys Res Comm*, 240, 324–328.

Maier DB & Kuslis ST. (1988) Human uterine luminal fluid volumes and prolactine levels in normal menstrual cycles. Am J Ostet Gynecol 159: 434-439.

Mann SE, Ricke EA, Torres EA, Taylor RN (2005) A novel model of polyhydramnios: amniotic fluid volume is increased in aquaporin 1 knockout mice. Am J obstet Gynecol. 192: 2041-4

Mann SE, Dvorak N, Gilbert H, Taylor RN (2006) Steady-state levels of aquaporin 1 mRNA expresion are increased in idiopatic polyhydramnios. Am J Obstet Gynecol. 194: 884-7

Mc Connel NA, Yunus RS, Gross SA, Bost KL, Clemens MG, Hughes FM Jr (2002) Water permeability of an ovarian astral follicle is predominantly transcellular and mediated by aquaporins. Endocrinology 143, 2905-2912

Medrano A, García-Gil N, Ramió L, Rivera MM, Fernández-Novell JM, Ramírez A, Peña A, Dolors Briz M, Pinart E, Concha II, Bonet S, Rigau T, Rodriguez-Gil JE (2006) Hexose-specificity of hexokinase and ADP-dependence of pyruvate kinase play important roles in the control of monosaccharide utilization in freshly diluted boar spermatozoa. Mol Reprod Dev 73(9): 1179-1194

Meng QX, Xu CM, Cai J, Huang HF (2005) Controlled ovarian hyperstimulation significantly decreases the expresión of aquaporin 3 in Mouse oocytes using semi-quantitative real-time PCR análisis. Acta Anat Sin 36, 381-385

Miglino MA, Ambrósio CE, dos Santos Martins D, Valverde Wenceslau C, Pfarrer C, Leiser R (2006) The carnivore pregnancy: The development of the embryo and fetal membranes. Theriogenology. 66: 1966-1702.

Mobasheri A, Wray S, Marples D. (2005) Distribution of AQP2 and AQP3 water channels in human tissue microarrays. J Mol Histol 36: 1-14

Moon C, Preston GM, Griffin CA, Jabs EW, Agre P (1993) The aquaporin-CHIP 28 gene: structure, organization and chromosomal localization. J Biol Chem. 268: 15772-15778.

Morriss F, Boyd R & Mahendran D (1994) Placenta Transport. In *The Physiology of Reproduction*, pp. 813–861. New York: Raven Press.

Mozzanega B, Mioni R, Granzotto M, Chiarelli S, Xamin N, Zuliani L, Sicolo N,March esoni D, Vettor R (2004) Obesity reduces the expression of GLUT4 in the endometrium of normoinsulinemic women affected by the polycystic ovary syndrome.Annals of the New York Academy of Sciences 1034: 364–374

Muekler M, Morris HR, Blench I, Allard WJ, Daldwin SA, Caruso C, Panico M, Lienhard GE, Lodish FH (1985) Sequence and structure of a human glucose transporter. Science. 229: 941-945.

N

Newman J (2008) Physics of the life science. Fisrt ed., Springer, New York.

Nielsen S, King LS, Christensen BM, Agre P (1997) Aquaporins in complex tissues. II. Subcellular distribution in respiratory and glandular tissues of rat. Am J Phys 273: C1549-C1561.

Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP (1997b) Specialized membrane domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. J Neurosci. 17:171-80.

Nielsen S, Froklaer J, Marples D, Kwon TH, Agre P, Knepper MA (2002) Aquaporins in the kidney: from molecules to medicine. Physiol Rev. 82: 205-244.

O

Offenberg H, Barcroft LC, Caveney A, Viuff D, Thomsen PD, Watson AJ (2000) mRNAs encoding aquaporins are present Turing murine preimplantational development. Mol Reprod Dev 57, 323-330.

Okada Y., Asahina T., Kobayashi T., Goto J., Terao T. (2001) Studies on the mechanism of edematous changes at the endometrial stroma for implantation. Semin Thromb Hemost 27: 67-77

Oliveira CA, Carnes K, Franca LR, Hermo L, Hess RA (2005) Aquaporin 1 and 9 are differentially regulated by estrógeno in the efferent ductule epithelium and inicial segmento f the epididymis. Biol Cell 97, 385-395

P

Page E, Winterfield J, Goings G, Bastawrous A, Upshaw-Earley J (1998) Water channel proteins in rat cardiac myocyte caveolae: osmolarity-dependent reversible internalization. AM J Physiol Heart Circ Physiol. 274: H1988-H2000.

Pantaleon M, Harvey MB, Pascoe WS, James DE, Kaye PL (1997) Glucose transporter GLUT3: ontogeny, targeting and role in the Mouse blastocyst. Proc Natl Acad Sci USA 94: 3795-3800.

Parrish JJ, Susko-Parrish JJ, First NL (1989) Capacitation of bovine sperm by heparin: Inhibitory effect of glucose and role of intracellular pH. Biol Reprod 41: 683-699.

Pastror-Soler N, Bagnis C, Sabolic I, Tyszkowski R, McKee M, Van Hoek A, Breton S, Brown D (2001) Aquaporin 9 expression along the male reproductive tract. Biol Reprod 65, 384-393

Pauerstein CJ and Eddy CA (1979) The role of the oviduct in reproduction; our knowledge and our ignorante. J Reprod Fertil 55, 223-229

Pinto AB, Carayannopoulus MO, Hoehn A, Dowd L, Molley KH (2002) Glucose transporter 8 expression and translocation are critical for murine blastocyst survival. Biology of reproduction 66(6): 1729-1733

Prat C, Blanchon L, Borel V, Gallot D, Herbet A, Bouvier D, Marceau G, Sapin V (2012) Ontogeny of aquaporins in human fetal membranes. *Biol Reprod*. 86, 48

Preston GM, Agre P (1991) Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. Proc Nat Acad Sci USA. 88: 11110-11114.

Preston GM, Carroll TP, Guggino WB, Agre P (1992) Appearance of water channels in Xenopus oocytes expressing red cell CHIP28 protein. Science. 256: 385-387.

Prieto-Martinez N, Vilagran I, Morató R, Rodriguez-Gil JE, Yeste M, Bonet S (2014). Aquaporins 7 and 11 in boar spermatozoa: detection, localisation and relationship with sperm quality. Reprod Feril and Dev 26, 663-672

Prieto-Martinez N, Morató R, Vilagran I, Rodriguez-Gil JE, Bonet S, Yeste M (2015) Aquaporins in boar spermatozoa. Part II: detection and localisation of aquaglycerolporin 3. Reprod Fertil Dev 10.1071/RD15164

Prieto-Martinez N, Morató R, Muiño R, Hidalgo CO, Rodriguez-Gil JE, Bonet S, Yeste M (2016) Aquaglycerolporins 3 and 7 in bull spermatozoa: identification, localisation and their relationship with sperm cryotolerance. Reprod Fertil Dev 10.1071/RD16077

R

Rigau T, Rivera M, Palomo MJ, Fernández-Novell JM, Mogas T, Ballester J, Peña A, Otaegui PJ, Guinovart JJ, Rodriguez-Gil JE (2002) Differential effects of glucose and fructose on hexose metabolism in dog spermatozoa. Reproduction 123(4) 579-591

Rogers S, Macheda ML, Docherty SE, Carty MD, Henderson M, Soeller WC, Gibbs EM, James DE, Best JD (20023) Identification of a novel glucose transporter-like proteing GLUT12. AM J Physiol Endocrinol Metab 282(3) E733-738

S

Saparov SM, Liu K, Agre P, Pohi P. (2007) Fast and selective ammonia transport by aquaporin-8. J Biol Chem 282: 5296–301.

Sasaki T, Minoshima S, Shiohama A, Shintani A, Shimizu A, Asakawa S, Kawasaki K, Shimizu N (2001) Molecular cloning of a member of the facilitative glucose transporter gene family GLUT11 (SLC2A11) and identification of transcription variants. Biochemical and Biophysical Research Communications 289, 1218–1224.

Senger PL (2003) Pathway to pregnancy and parturition. 2nd Revised ed. llman, Washington: Current Conceptions Inc.

Sha XY, Xiong ZF, Liu HS, Di XD, Ma TH (2011) Maternal-fetal fluid balance and aquaporins: from molecule to physiology. Acta Pharmacologica Sinica. 32:716-720.

Shanahan CM, Connolly DL, Tyson KL, Cary NR, Osbourn JK, Agre P, Weissberg PL (1999) Aquaporin-1 is expressed by vascular smooth muscle cells and mediates rapid water transport across vascular cell membranes. J Vasc Res. 36:353–62.

Sheepers A, Joost HG, Schürmann A (2004) The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. JPEN J Parenter Enteral Nutr 28(5) 364-371

Shepherd PR & Kahn BB (1999) Glucose transporters and insulin action – implications for insulin resistance and diabetes mellitus. New England Journal of Medicine 341, 248–257.

Shioji M, Fukuda H, Kanzaki T, Wasada K, Kanagawa T, Shimoya K, Mu J, Sugimoto Y, Murata Y (2006) Reduction of aquaporin-8 on fetal membranes under oligohydramnios in mice Licking prostagrandin F2 alpha receptor. J Obstet Gynaecol Res. 32: 373-8

Shirazi-Beechey SP, Hirayama BA, Wang Y, Scott D, Smith MW, Wright EM (1991) Ontogenic development of lamb intestinal sodium-glucose co-transporters is regulated by diet. J Physiol 437: 688-708

Sciullo E, Cardellini G, Baroni M, Torresi P, Mazziotti P, Pozzilli P, Fallucca F (1997) Glucose transporters (GLUT 1, GLUT 3) mRNA in human placenta of diabetic and non-diabetic pregnancies. Ann Ist Super Sanita. 33: 361-5.

Simpson IA, Dwyer D, Matilde D, Moley KH, Travis A, Vannucci SJ. 2008. The facilitative glucose transporter GLUT3: 20 years of distinction. Am J Physiol Endocrinol Metab 295: E242-E253

Skowronski MT (2010) Distribution and quantitative changes in amounts of aquaporin 1, 5 and 9 in the pig uterus during the estrous cycle and early pregnancy. Reproductive Biology and Endocrinology 8:109.

Smith BL, Agre P (1991) Erythrocyte Mr 28000 transmembrane protein exists as a multisubunit oligomer similar to channel proteins. J Biol Chem. 266: 6407-6415

Suzuki-Toyota F, Ishibashi K, Yuasa S (1999) Immunohistochemical localization of a water channel, aquaporin 7 (AQP7), inthe rat testis. Cell Tissue Res 259, 279-285

T

Tani T, Koyama Y, Nihei K, Hatakeyama S, Ohshiro K, Yoshida Y, Yaotia E, Sakai Y, Hatakeyama K, Yamamoto T (2001) Immunolocalization of aquaporin-8 in rat digestive organs and testis. Arch Histol Cytol 64, 159-168.

Thoroddsen A, Dahm-Kähler P, Lind AK, Weijdegard B, Lindenthal B, Müller J, Brännström M (2011) The water permeability chennels aquaporins 1-4 are differentially expressed in granulose and theca cells of the preovulatory follicle during precise stages of human ovulation. J Clin Endocrinol Metab 96(4): 1021- 8

Tsukaguchi H, Shayakul C, Berger UV, Mackenzie B, Devidas S, Guggino WB, van Hoek AN, Hediger MA (1998) Molecular characterization of a broad selectivity neutral solote channel. J Biol Chem 273, 24737-24743

Turner RJ & Silverman M (1977) Sugar uptake into brush borders vesicles from normal human kidney. Proc Natl Acad Sci USA 74: 2825-2829

U

Uldry M, Ibberson M, Horisberger JD, Chatton JY, Rieder BM, Thorens B (2001) Identification of a mammalian H-myoinositol symporter expressed predominantly in the brain. EMBO J 20: 4467-4477.

Uldry M & Thorens B (2004) The SCL2 family of facilitated hexose and polyol transportes. Pflugers Arch – Eur J Physiol 447: 480-489

Umenishi F., Verkman AS., Gropper MA (1996) Quantitative análisis of aquaporin mRNA expresion in rat tissues by RNase protection on assay. DNA Cell Biol. 15:475-80

V

Verco CJ (1994) Fallopian tube anatomy, microanatomy, microcirculation and counter-current Exchange. In Grudzindkas, JG, Chapman, MG, Chard, T *et al.,* (eds) The fallopian tube: Clinical and surgical aspects. Springer-Verlag, New Yourk, NY, USA, pp. 3-15.

Von Wolf M, Ursel S, Hahn U, Steldinger R, Strowitzki T (2003) Glucose transporter proteins (GLUTs) in human endometrium: expression, regulation and function throughout the menstrual cycle and in early pregnancy. Journal of endocrinology and metabolism 88: 3885 - 3892

W

Wang S, Kallichanda, N, Song, W, Ramirez BA, Ross MG, (2001) Expression of aquaporin-8 in human placenta and chorioamniotic membranes: Evidence of molecular mechanism for intramembranous amniotic fluid resorption. Am J Obstet Gynecol 185: 1226-1231.

Watson AJ (1992) The cell biology of blastocysts development. Front Biosci 6, D708-D730.

Welch RD & Gorski J (1999) Regulation of glucose transporters by estradiol in the immature rat uterus. Endocrinology 140 3602–3608

Wheeler T. J., Hinkle P. C. (1981) Kinetic properties of the reconstituted glucose transporter from human erythrocytes. J Biol Chem 256(17) 8907-8914.

Wong PY & Yeung CH (1978) Absorptive and secretory functions of the perfused rat cauda epididymis. J Physiol 275, 13-26.

Wooding FBO, Dantzer VB, Klisch K, Jones CJ, Forhead AJ (2007) Glucose transporter 1 localisation throughout pregnancy in the carnivore placenta: Light and electron microscope studies. Placenta 28: 453-464.

Wu X, Freeze HH (2002) GLUT14, a duplicon of GLUT3, is specifically expressed in testis as alternative splice forms. Genomics 80: 553-557.

X

Xing AY, Challier JC, Lepercq J, Caüzac M, Charron MJ, Girard J, Hauguel-de Mouzon S (1998) Unexpected expression of glucose transporter 4 in villious stromal cells of human placenta. J Clin Endocrinol Metab 83(11): 4097 - 101

Y

Yamaguchi M, Sakata M, Ogura K, Miyake A (1996) Gestational changes of glucose transporter gene expression in the mouse placenta and decidua. J Endocrinol Invest. 19(8): 567-9.

Yasui M, Hazama A, Kwon TH, Nielsen S, Guggino WB, Agre P. (1999) Rapid gating and anion permeability o fan intracellular aquaporin. Nature. 402: 184-7.

Z

Zhou Q, Clarke L, Nie R, Carnes K, Lai LW, Lien YH, Verkman A, Lubahn D, Fisher JS, Katzenellenbogen BS, Hess RA (2001) Estrogen activation and male fertility: roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. Proc Natl Acad Sci U S A 98, 14132-14137.

Zhu XQ, Jiang SS, Zhu XJ, Zou SW, Wang YH, Hu YC (2009) Expression of aquaporin 1 and aquaporin 3 in fetal membranes and placenta in human term pregnancies with oligohydramnios. Placenta 30: 670-676