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EFECTOS DE UNA DIETA SUPLEMENTADA CON BIOTINA SOBRE LOS MECANISMOS QUE PARTICIPAN EN LA PROLIFERACIÓN DE LAS ESPERMATOGONIAS DE RATÓN

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INDICE

ABSTRACT

Besides its physiological role as a carboxylase prosthetic group, pharmacological concentrations of biotin influence different systemic functions. Supplements containing pharmacological concentrations of biotin are commercially available. The mechanisms by which biotin at pharmacological concentrations exerts its action have been the subject of multiple investigations, particularly for biotin's medicinal potential and wide use for cosmetic purposes. Several studies have reported that biotin supplementation increases cell proliferation; however, the mechanisms involved in this effect have not yet been characterized. In a previous study, we found that a biotin-supplemented diet increased spermatogonia proliferation. The present study was focused on investigating the molecular mechanisms involved in biotin-induced testis cell proliferation. Male BALB/cAnNHsd mice were fed a control or a biotin-supplemented diet (1.76 or 97.7 mg biotin/kg diet) for eight weeks. Compared with the control group, the biotin-supplemented mice presented augmented protein abundance of the c-kit-receptor and pERK1/2^{Tyr204} and pAKT^{Ser473}, the active forms of ERK/AKT proliferation signaling pathways. No changes were observed in the testis expression of the stem cell factor and in the serum levels of the follicle-stimulating hormone. Analysis of mRNA abundance found an increase in cyclins *Ccnd3*, *Ccne1*, *Ccna2*; Kinases *Cdk4, Cdk2; and E2F;* and *Sp1 & Sp3* transcription factors. Decreased expression of cyclin-dependent kinase inhibitor 1a (*p21*) was observed but not of *Cdkn2a* inhibitor (*p16*), it did not change significatively. The results of the present study identify, for the first time, the mechanisms associated with biotin supplementation-induced cell proliferation, which raises concerns about the effects of biotin on male reproductive health because of its capacity to cause hyperplasia, especially because this vitamin is available in large amounts without regulation.

RESUMEN

La biotina, además de su función como grupo prostético de las carboxilasas, esta vitamina modifica diversas funciones biológicas al administrarse en concentraciones farmacológicas. Existen comercialmente suplementos vitamínicos que contienen concentraciones farmacológicas de biotina. Los mecanismos moleculares y vías de señalización por los que la biotina ejerce sus efectos farmacológicos en cosmetología, inmunología y en diabetes mellitus han sido sujetos de estudios. Se ha documentado que concentraciones farmacológicas de biotina aumenta la proliferación celular; sin embargo, las vías de señalización molecular que participan en estos efectos no han sido establecidas. En estudios anteriores encontramos que una dieta suplementada 56 veces más comparada con la dieta control (1.76 o 97.7 mg biotina/kg) aumentó la proliferación de las espermatogonias. Investigamos las vías de señalización molecular que participan en la proliferación celular. Por lo que ratones macho de la cepa BALB/cAnNHsd se alimentaron con una dieta control vs una dieta suplementada con biotina (1.76 o 97.7 mg biotina/kg) durante 8 semanas. Los resultados mostraron que los ratones del grupo suplementado con biotina aumentan la cantidad de transcritos de las ciclinas *Ccnd3*, *Ccne1*, *Ccna2*; de las cinasas *Cdk4, Cdk2* y los factores de transcripción *E2F, Sp1* y *Sp3,* moleculas encargados de la progresión del ciclo celular. Así como la disminución significativa del transcrito del inhibidor dependiente de ciclinas tipo 1 (*p21*), sin cambio significativo en la expresión del transcrito del inhibidor *p16*. A nivel de proteína, se observó aumento en la concentración de proteínas de las vías de señalización MAPK y AKT encargadas de activar los complejos ciclinas/cinasas; así como aumento de la proteína del receptor transmembranal CKIT. Interesantemente, no hubo modificación en la concentración de la Hormona Foliculo Estimulante FSH en suero ni cambios de la proteina del Factor de Células Troncales/Stem SCF, ambas moléculas participan en el eje hipotálamo/hipófisis/gónada; por lo que el efecto proliferativo de la biotina sobre el tejido testicular no es una señalización endocrina sino es *in situ* mediante el incremento de proteínas de las vías de señalización MAPK y AKT.

Los resultados de este estudio identifican los mecanismos moleculares asociados con el efecto proliferativo celular por la administración oral de la biotina en concentraciones farmacológicas; que tiene la capacidad de modificar la morfología típica de la estructura del tejido testicular, así como un alcance potencial en la alteración fisiología del tejido testicular en modelos animales; queda por realizar estudios para complementar información sobre las alteraciones que podrían comprometer la anatomía y la fisiología testicular por el consumo o administración desmedido de biotina.

ABREVIATURAS.

AKT, Protein cinasa B (PKB).

Ccna2, Ciclina A2.

***Ccne1**,* Ciclina E.

Ccnd3, Ciclina D3.

Cdk2, cinasa dependiente de ciclina 2.

Cdk4, cinasa dependiente de ciclina 4.

E2F, Factor transcripcional E2F.

ERK1, cinasa regulada por señales extracelulares tipo 1.

ERK2, cinasa regulada por señales extracelulares tipo 2.

FSH, Hormona Folículo Estimulante.

GAPDH, Gliceraldehído-3-fosfato deshidrogenasa.

p16, inhibidor de cinasa dependiente de ciclina tipo 2A gene.

p21, inhibidor de cinasa dependiente de ciclina tipo 1.

pAKT^{Ser473}, AKT fosforilado en serina 473.

pAKT^{Thr308}, AKT fosforilado en Treonina 308.

pERK1/2^{Tyr204}, cinasa regulada por señales extracelulares tipo 1-2 fosforilada.

SCF, Factor de células troncales.

ES, error estándar.

***Sp1**, proteína específica* 1.

***Sp3**, proteína específica* 3.

Tuba1α, alfa tubulina.

BIOTINA: GENERALIDADES DE LA BIOTINA.

La biotina es una vitamina hidrosoluble cuya función es participar como grupo prostético de las enzimas carboxilasas, las cuales regulan el metabolismo de carbohidratos, lípidos, aminoácidos (Zempleni, 2005). El nombre químico de la biotina es ácido hexahidro-2-oxo-1H-tieno-[3,4-d] imidazol-4-pentoico; es un ácido monocarboxílico con peso molecular de 244.31 g/mol, su estructura química está conformada por un anillo de tetrahidrotiofeno, grupo ureido (-N-CO-N-), azufre, y una cadena de ácido valérico que le confiere su naturaleza ácida con pKa=4.4; es estable al calor, soluble en agua (0.02% p/v) y etanol (0.08% p/v) y susceptible a la oxidación (Dakshinamurti & Chauhan, 1989) Figura A.

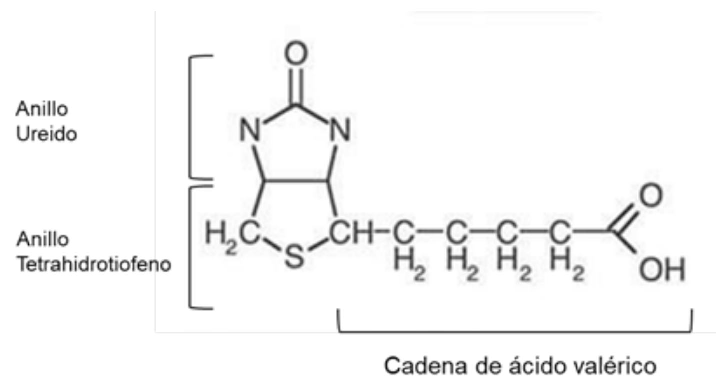

Figura A. Estructura química de la vitamina Biotina.

LA BIOTINA EN LOS ALIMENTOS

Los humanos obtienen la biotina en la dieta principalmente, y en menor cantidad por la síntesis de *novo* mediante el metabolismo de bacterias intestinales (McMahon *et al*. 2002; Said, 2012). La ingesta recomendada diaria en adulto es de 30 µg y 25 µg para niños (Institute of Medicine & Nutrition, 1998). Esta vitamina se encuentra ampliamente distribuida en diferentes alimentos; por ejemplo, en la leche, el hígado, la yema de huevo, los cereales y vegetales (Ensminger, 1993; Said, 2012). La biodisponibilidad de la biotina varía de una fuente a otra, siendo el 100% en el maíz y sólo del 5% en el trigo (Said, 2012). En la dieta, puede estar libre o unida covalentemente a proteínas mediante un residuo de lisina.

DIGESTIÓN Y ABSORCIÓN DE LA BIOTINA

Las enzimas peptidasas y proteasas son las encargadas de la digestión de los compuestos que contienen biotina para la obtención de biocitina, el compuesto más simple formado por biotina y lisina. La biocitina es catabolizada en biotina y lisina por la enzima biotinidasa que se encuentra en el jugo pancreático, permitiendo su absorción por los enterocitos a través del Transportador transmembranal Múltiple de Vitaminas Dependiente de Sodio (SMVT). SMVT es el producto del gen *SLC5A6* y está conformado por 634 aminoácidos con un peso molecular de 69 kD; localizado en la porción apical de los enterocitos, que realiza un transporte simporte electrogénico, a favor de un gradiente de Sodio y dependiente de temperatura (Said, 2009).

SMVT es responsable de la translocación de vitaminas y otros cofactores esenciales como la biotina, el ácido pantoténico y el ácido lipoico (Prasad et al., 1997, 1998, 1999). La constante de afinidad (Km) de SMVT1 para el ácido pantoténico y lipoico es de 1~5 μM la cual corresponde a la concentración de ácido pantoténico circulante (0.886-4.59 μM). La Km de SMVT1 para la biotina que es de ~15 μM, siendo la concentración de biotina libre de ~0.5-2 nM. (Combs GF.1992), por lo que la ingesta de biotina puede ser inhibida por el ácido pantoténico o el ácido lipoico. SMVT transporta biotina, ácido lipídico y ácido pantoténico junto con el sodio, con una estequiometría de 2:1 sodio-sustrato, por cada molécula de vitamina que entra a la célula, dos iones de sodio son cotransportados al interior de esta (Prasad et al. 1998; Prasad et al. 1997). Una vez que la biotina cruza la membrana apical, su salida del enterocito también se da a través del transportador. El transportador SMVT media la absorción intestinal de biotina y también es el responsable de la captación de biotina en el hígado, en los tejidos periféricos y para la reabsorción renal (Zempleni, 2009), así como en en otros tejidos como cerebro, corazón y placenta (Said, 2012).

Se ha proporcionado evidencia de que el *Transportador de monocarbolxilatos (MCT1)* también participa en la captación de biotina en algunos linajes celulares, como las células mononucleares de sangre periférica y los queratinocitos (Daberkow et al 2003). Este transportador es un miembro de la familia de transportadores de monocarboxilatos que facilitan la ingesta de biotina con una Km tres veces menor a la usada por SMVT, además de que no es inhibido competitivamente por el ácido pantoténico o lipoico.

TRANSPORTE DE BIOTINA HACIA LOS TEJIDOS

Después de absorberse en el intestino, la biotina pasa al plasma, la cual se encuentra libre en un 81 %, o bien unida a proteínas de manera covalente en un 12 % y unida reversiblemente en aproximadamente un 7 % (Mock et al. 2005). Se ha propuesto que SMVT1 es la principal proteína de transporte de la biotina (Zempleni et al. 2005). La biotina es captada de la circulación por los órganos periféricos. La cantidad de biotina que se encuentra intracelularmente ya sea en forma unida o libre, depende del balance entre su captación, el equilibrio entre su liberación de proteínas biotiniladas e incorporación a las mismas, así como de su catabolismo (Mock et al. 2005).

TRANSPORTE INTRACELULAR DE BIOTINA.

La biotina internalizada en la célula se encuentra en la mitocondria y en el citoplasma, y en menor cantidad en el núcleo con un 0.7% (Zempleni, 2005). El mecanismo de transporte de biotina hacia la mitocondria se ha estudiado utilizando mitocondrias aisladas de hepatocitos, y se ha sugerido que es dependiente de pH. Se creé que la biotina entra al espacio intramitocondrial en su forma protonada (neutra), para posteriormente disociarse en su forma aniónica (pKa = 4.5) en el pH alcalino de la mitocondria (Said, 2012).

FUNCIÓN DE LA BIOTINA

En los mamíferos, la biotina actúa como como grupo prostético de las carboxilasas, enzimas que participan en el metabolismo intermediario. Estas carboxilasas dependientes de biotina son: El piruvato carboxilasa (PC, EC 6.4.1.1), la propionil-CoA carboxilasa (PCC, EC 6.6.1.3), la β-metilcrotonil-CoA carboxilasa (MCC, EC 6.4.1.4) y la acetil-CoA-carboxilasa (ACC, EC 6.4.1.2). de las cuales existen dos isoformas: la acetil-CoA-carboxilasa 1 presente en el citosol, y la acetil-CoA-carboxilasa en la mitocondria. Estas enzimas catalizan la incorporación de bicarbonato, en forma de un grupo carboxilo, a sus respectivos sustratos (Mock et al. 2005).

La biotina se une a las carboxilasas inactivas (apocarboxilasas) mediante una reacción dependiente de ATP, catalizada por la enzima holocarboxilasa sintetasa (HCS) (Figura B). En el primer paso de la reacción se forma un intermediario de biotina y ATP, el biotinil-5'- adenilato. Posteriormente, el grupo carboxilo de la cadena de ácido valérico de la biotina y el grupo ε-amino de una lisina en una región específica de la apocarboxilasa forman un enlace covalente. Estas regiones catalíticas, las apocarboxilasas contienen residuos de aminoácidos altamente conservados entre especies. Una vez unidas a la biotina, las holocarboxilasas son capaces de formar un intermediario carboxifosfato en presencia de HCO3- y ATP, para trasladar el CO_2 de la biotina al sustrato (Said, 2012).

Grupo prostético de las carboxilasas:	
Piruvato carboxilasa	Enzima encargada de catalizar la conversión de piruvato en oxalacetato, implicada en la gluconeogénesis.
Propionil-CoA carboxilasa	Enzima ligasa que cataliza la reacción de carboxilación de propionil-CoA que participa en el metabolismo de ácidos grasos.
β-metilcrotonil-CoA carboxilasa	Enzima encargada de catalizar la reacción de carboxilación del ácido 3-metilcrotónico a ácido 3-metilglutacónico, el cual participa en el metabolismo del aminoácido esencial leucina.
Acetil-CoA-carboxilasa	Cataliza la reacción de adición de un grupo bicarbonato al acetato para obtener malonato. Regula la biosíntesis de los ácidos grasos y su oxidación.

CATABOLISMO DE LA BIOTINA

La biotina unida a las holocarboxilasas se libera en forma de biocitina por degradación proteolítica de la holocarboxilasas. Esta biocitina puede ser reutilizada por otras carboxilasas, o catalizarse hacia sulfóxido de biotina o Bisnorbiotina y posteriormente excretarse en orina (Said, 2012). La vida media de la biotina en la fase de eliminación lenta (21.9 ± 13.6h) coincide con el rompimiento de las carboxilasas biotiniladas (Zempleni, 2005).

TRANSPORTE Y METABOLISMO DE LA BIOTINA.

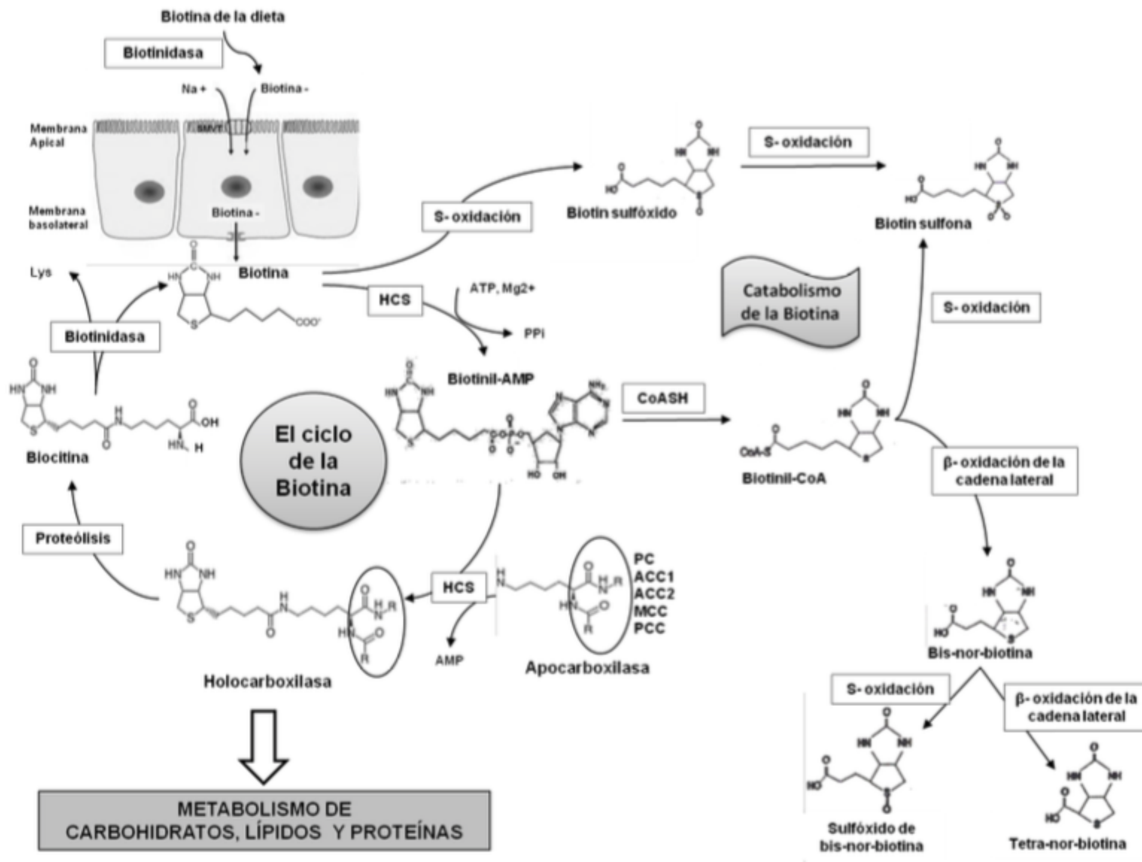

Figura B. Transporte y metabolismo de la biotina. La biotina ingerida por la dieta es transportada a través de los enterocitos mediante transportadores SMVT, la enzima holocarboxilasa sintetasa (HCS) biotiniliza las apoenzimas carboxilasas mediante dos pasos para activar su función y participar en el metabolismo de carbohidratos, lípidos y proteínas; posteriormente, la biotinidasa cataliza la liberación de biotina.

EFECTOS DE CONCENTRACIONES FARMACOLÓGICAS DE BIOTINA

Varios estudios han encontrado que, en dosis farmacológicas de biotina, que van de 50 a 700 veces sus requerimientos diarios, la biotina es capaz de modificar diversas funciones biológicas. Entre estas funciones se encuentran el metabolismo de los triglicéridos y de la glucosa, la reproducción, la producción de leche y funciones del sistema inmune, la proliferación celular y cambios en algunos tejidos, tales como pancreático, hepático y testicular (Revisado en Riverón-Negrete et al. 2017). Estos efectos son diferentes al de su acción como grupo prostético de las carboxilasas. Se ha propuesto que el mecanismo por el que se producen los efectos farmacológicos de la biotina está asociado con cambios en la expresión de genes mediante la acción de la guanilato ciclasa.

Estudios en los años 1960s' mostraron que la biotina en miligramos, poseé efectos diferentes a los de su función como grupo prostético de las carboxilasas, tal como cambio en la actividad de la glucocinasa. Dakshinamurti y colabaradores fueron los primeros en demostrar que las concentraciones farmacológicas de biotina afectan a las proteínas no relacionadas con las enzimas que participan en el metabolismo de la biotina (Dakshinamurti y Cheah, 1968; Dakshinamurti y Ho, 1970). En la década de 1960, Dakshinamurti y Cheah-Tan encontraron que una inyección aguda de biotina (1-2 mg/kg) aumentaba la actividad de la glucoquinasa hepática en ratas diabéticas con aloxano y en ratas no diabéticas en ayunas; este efecto también se observó en hepatocitos de rata cultivados (Spence y Koudelka, 1984). Posteriormente, se descubrió que la acción de la biotina sobre la glucoquinasa aumentaba la transcripción (Chauhan, J.; Dakshinamurti, K., 1991). Estos descubrimientos fueron los primeros en discernir algunos de los efectos de la biotina en la expresión génica.

Estudios posteriores, demostraron que estos efectos son regulados mediante la expresión transcripcional, traduccional y modificación post-traduccional (Riverón-Negrete y Fermández Mejía 2017). El mecanismo molecular de la acción farmacológica de la biotina sobre la expresión de genes está asociado con la vía de señalización modulada por la guanilato-ciclasa soluble, el GMPc y la proteíncinasa G (GC/GMPc/PKG). (Vilches-Flores et al., 2010 A; Aguilera-Méndez A et al., 2012; Boone-Villa D et al., 2015; Moreno-Méndez E et al., 2018).

En 1982, Vesely encontró que la biotina 1 mM, o su análogo (+)-biotina-p-nitrofenil éster, aumentó la actividad de la guanilato ciclasa de dos a tres veces en homogeneizados de hígado, corazón, riñón, cerebelo y colón de rata (Vesely DL. 1982). En 1984, el mismo grupo descubrió que los análogos de biotina azabiotina, bisnorazabiotina, carbobiotina e isoazabiotina también podían mejorar la actividad de la guanilato ciclasa en homogeneizados de tejido (Vesely D., 1984). El mismo año, los estudios de Spence y Kouldeka encontraron que el cultivo de hepatocitos aislados con biotina [10 nM a 1 mM, respuesta máxima a 1 mM] aumentaba los niveles de cGMP (Spence J., 1984). En cultivos de células, otros estudios, usando bloqueadores e inhibidores, identificaron que la vía de señalización de cGMP/PKG media la transcripción de carboxilasas, holocarboxilasa sintetasa, el transportador multivitamínico dependiente de sodio (Solorzano-Vargas, R 2002; Pacheco-Alvarez D et al., 2005), el receptor de asialoglicoproteína (De La Vega, L. 2000) y el receptor de insulina (De La Vega, L. 2000) en respuesta a biotina 1 mM. En islotes pancreáticos aislados de ratas, la expresión de ARNm de glucoquinasa inducida por biotina dependía de la guanilato ciclasa (Vilches-Flores, A 2010). *In vivo*, 8 semanas de suplementación con biotina en la dieta (97,7 mg/kg de dieta) aumentó el cGMP en los tejidos hepático y adiposo (Aguilera-Mendez, A 2012; Boone-Villa, D 2015).
La biotinilación de histonas ha sido un mecanismo propuesto en la modificación de la expresión transcripcional; sin embargo, Zempleni y colaboradores mencionan que es fenómeno controversial y que a pesar de ser natural se presenta raro en células humanas; por lo que no ha cobrado trascendencia en la publicación de artículos recientes (Kuroishi et al., 2011; Riverón-Negrete y Fernández-Mejía 2017).

EFECTOS DE CONCENTRACIONES FARMACOLÓGICAS DE BIOTINA SOBRE FUNCIONES BIOLÓGICAS.

En células mononucleares de sangre periférica obtenida de humanos sanos, la abundancia del ARNm de interferón-gamma e interleucina-1beta se incrementaron aproximadamente 4.5 veces después de la administración de 1.2 mg biotina/día por 21 días (Wiedmann S., et al. 2003).

En modelos animales, tales como ratas con hipertensión, la ingesta de biotina durante 8 semanas (1.2 mg/kg peso corporal) disminuyó la presión arterial sistólica, el grosor de las arterias coronarias, y la incidencia de infarto inducido (Watanabe-Kamiyama M., et al. 2008). En ratones, estudios *in vivo* encontraron que la suplementación con biotina disminuyó la inflamación producida por alergia al níquel mediante la producción de interleucina-1beta (Kuroishi T., et al. 2009). En vacas, se encontró que la suplementación de la dieta con biotina (0, 10, 20 mg al día) incrementó la producción de leche (Zimmerly, C., et al. 2001). También, se ha reportado que reduce el número de retención de la placenta y endometritis puerperal con la administración de 10 mg de biotina al día en vacas (Kinal S., et al. 2011).

EFECTOS DE CONCENTRACIONES FARMACOLÓGICAS DE BIOTINA SOBRE EL METBOLISMO DE LA GLUCOSA Y DE LOS LÍPIDOS.

Investigaciones diversas han encontrado que la biotina en concentraciones farmacológicas disminuye la hipertrigliceridemia y la intolerancia a la glucosa tanto en humanos como en animales de laboratorio (Riveron-Fernández 2017), lo que confiere gran importancia al estudio de los mecanismos de acción de la biotina por su uso potencial en la prevención y/o tratamiento de la diabetes tipo-2 y el síndrome metabólico.

Estudios realizados por nuestro grupo de investigación en ratones macho de la cepa Balb/cAnN alimentados con una dieta que contiene 56 veces más la cantidad de biotina con respecto a la dieta control durante 8 semanas posteriores al destete, mostraron efectos favorables en la homeostasis de la glucosa, tales como la disminución más rápida de la glucosa durante su prueba bioquímica de tolerancia, el incremento de la secreción de insulina *in vivo* e *in vitro*, el aumento de la expresión de genes relacionados con la síntesis y secreción de la insulina (Lazo de la Vega-Monroy et al. 2013). En ratones con hiperglucemia inducida por daño pancreático con estreptozotocina, la administración de 15 mg de biotina/kg de peso durante 12 días consecutivos, disminuyó la glucosa sanguínea y redujo el daño renal y hepático producido por la hiperglucemia (Aldamash et al 2015; Aldamash et al., 2016). En pacientes con diabetes, se ha reportado que la administración de biotina en el orden de miligramos disminuye la hiperglucemia (Coggeshall et al. 1985; Maebashi et al. 1993; Koutsikos et al. 1996, Singer et al. 2006).

La administración de 5 mg de biotina cada 24 horas durante 4 semanas en pacientes con ateroesclerosis e hipercolesterolemia disminuyó la concentración de colesterol total (Dukusova y Krivoruchenko, 1972). En nuestro laboratorio, encontramos que el tratamiento con 5 mg de biotina cada 8 horas al día durante 4 semanas, disminuyó la concentración de triglicéridos en suero y las lipoproteínas de baja densidad tanto en pacientes diabéticos como en individuos no diabéticos con hipertrigliceridemia

(Revilla *et al.,* 2006). En ratones sanos, la administración de una dieta con biotina (97.7 mg/kg) durante 8 semanas disminuyó los niveles séricos de triglicéridos y de ácidos grasos en comparación con el grupo de ratones con dieta control (1.76 mg//kg de dieta). Los mecanismos moleculares mediante los cuales se producen estos efectos están asociados con un decremento en la expresión de genes, factores lipogénicos y en la fosforilación de proteínas reguladoras de estas vías. (Larrieta et al. 2010, Aguilera-Méndez y Fernández Mejía 2012, Boone et al., 2015).

EFECTO DE CONCENTRACIONES FARMACOLÓGICAS DE BIOTINA EN L MORFOLOGÍA TISULAR.

Estudios en nuestro laboratorio encontraron que en el mismo modelo experimental en donde se encontró aumento de la secreción de insulina, disminución de los niveles de glucosa y triglicéridos por efecto de la dieta suplementada con biotina (descritos en las secciones anteriores); también modifica el tamaño y la arquitectura típica de los islotes pancreáticos, aumenta el porcentaje de células alfa localizadas hacia el centro del islote de los ratones suplementados con la dieta de biotina (Lazo de la Vega-Monroy et al., 2013).

En el hígado de ratones alimentados con una dieta que contiene 56 veces más la cantidad de biotina con respecto a la dieta control durante 8 semanas los resultados revelaron incrementos significativos en el porcentaje de hepatocitos binucleados y nucleomegalia, dilatación de sinusoides y aumento en la vascularización. Interesantemente, este cambio estructural del hígado no se vio reflejado en incrementos de marcadores de estrés oxidante, ni en enzimas de escape indicativas de daño hepático (Riverón-Negrete et al., 2016).

En otro estudio encontramos que esa misma dieta durante 9 semanas produjo cambios en la estructura de los ovarios, disminuyendo el número de folículos primarios y de folículos de Graaf (Baez-Saldaña *et al*., 2009). También, en otros estudios el tratamiento agudo con biotina (100 mg/kg de peso corporal) indujo atrofia en el cuerpo lúteo en ratas (Paul *et al.,* 1973).

EFECTO DE CONCENTRACIONES FARMACOLOGICAS DE BIOTINA SOBRE LA PROLIFERACIÓN CELULAR

Se conoce poco de los efectos de la biotina sobre la proliferación celular, así como de los mecanismos moleculares participantes. En la línea celular de leucemia linfoblástica de células T, Jurkat, cultivadas y suplementadas con concentraciones farmacológicas de biotina durante dos semanas, se observó mediante incorporación de 5'bromo-2'-deoxyuridina, aumento transitorio de la proliferación celular (Manthey et al., 2002). Igualmente, en un estudio posterior en la línea celular de coriocarcinoma JAr, se observó un aumento en la incorporación de timidina tritiada [^{3}H] en respuesta a concentraciones farmacológicas de esta vitamina (Crisp et al., 2004). Sin embargo, no existen estudios sobre mecanismos moleculares o vías de señalización participantes en la proliferación celular. Sin embargo, en otros estudios se encontró que concentraciones farmacológicas de biotina disminuyen (Zempleni et al 2001) o no afectan la proliferación celular (Valenciano et al. 2002).

Estudios recientes en nuestro laboratorio encontraron que la administración de una dieta conteniendo concentraciones farmacológicas de biotina durante una semana después del destete, aumentó la proliferación de las células del islote observándose mayor número de células beta y mayor presencia del marcador de proliferación celular Ki-67 (Tixi-Verdugo W. *et al*., 2017).

Ha sido escasa la información sobre los mecanismos moleculares por los cuales las concentraciones farmacológicas de biotina modifican la proliferación y el ciclo celular. Estudios de microarreglos en sangre periférica de células mononucleadas aisladas de individuos sanos que ingirieron suplementos de biotina conteniendo (8.8 μmol/día por 21 días) reveló que, basado en la localización celular un gran número de genes, pertenecían a moléculas nucleares (Wiedmann y cols., 2004). La clasificación basada en las funciones biológicas encontró que entre los más prominentes se encontraban los relacionados con el ciclo celular y la proliferación (Wiedmann y cols., 2004). Interesantemente, transcritos mayormente modificados

fueron los relacionados con las señales transduccionales; sin embargo, este estudio no investigó la identidad de estos mensajeros. Otros estudios han sugerido que la biotina ejerce sus efectos sobre la proliferación modificando la biotinilación de histonas (Crisp et al 2004 Stanley, J, 2001). Sin embargo, este mecanismo es controversial (Bailey et al 2008; Heal et al 2009), siendo hasta el momento el conocimiento más aceptado que la biotinilación de histonas es un mecanismo que sucede en proporción baja (Kuroishi et al 2011).

LOS EFECTOS DE LA BIOTINA EN CONCENTRACIONES FARMACOLÓGICAS SOBRE LA PROLIFERACIÓN Y LA MORFOLOGÍA DEL TEJIDO TESTICULAR.

Con base en los hallazgos del efecto de la biotina sobre la morfología tisular, se investigó si en el mismo modelo experimental de ratones alimentados con una dieta suplementada (56 veces más la cantidad de biotina con respecto a la dieta control) durante 8 semanas afectaban al testículo, un tejido altamente proliferativo y sensible a tóxicos y medicamentos. Los resultados revelaron cambios, encontrándose que en el testículo se produjo: 1) Desorganización celular, 2) Aumento de vacuolas, 3) Hipertrofia de las células de Leydig, 4) Aumento en el número de espermatogonias y 5) Pérdida de la circularidad de los túbulos seminíferos; interesantemente, sin cambio significativo en los pesos de los testículos entre el grupo control y suplementado: 183 $\pm$ 6.45 vs 187 $\pm$ 3.12 mg (Pasten-Hidalgo et al., 2020). En el espermatozoide se encontró con un aumento en el doblamiento en la pieza media del flagelo y disminución en su movimiento progresivo, pero sin encontrarse cambios en la cantidad espermática. Estos resultados contrastan con los estudios *in vitro* mencionados en párrafos anteriores por Kalthur *et al*., 2015 y Salian 2019, quienes observaron que en muestras de semen humano incubadas *in vitro* con biotina se aumentó la motilidad de los espermatozoides y hubo mayor sobrevivencia con respecto a las muestras control, sin que el tratamiento dañase la integridad del ADN o se produjese reacción acrosomal temprana. El efecto diferente de la biotina entre los estudios de Kalthur et al. (2015) y nuestra investigación podría estar relacionado con la concentración de biotina.

Nuestros resultados mostraron un incremento del marcador de proliferación Ki-67 mediante ensayos de inmunodetección en tejido testicular de ratones suplementados con biotina (Pastén-Hidalgo 2020). También, se observaron alteraciones en la morfología del tejido testicular: que incluía desorganización de los túbulos seminíferos y alteración en su a forma circular típica, incrementos del tamaño del tejido intersticial, así como lo que demostró que, al igual que en el islote, concentraciones farmacológicas de biotina genera alteraciones en la morfología y

aumentos de la proliferación celular en el testículo. Interesantemente, se ha encontrado acumulación de biotina en el tejido testicular de ratones suplementados, lo que indica su capacidad para atravesar la barrera hematotesticular. Con base en estos resultados, el efecto de la biotina sobre la proliferación podría ser mediante mecanismos celulares *in situ*, independiente de alguna señalización hormonal o cambios en el eje hipotálamo-hipofisario-gónada (testículo), sin embargo, queda por comprobar cómo y cuáles mecanismos participan en la proliferación del tejido testicular.

TESTÍCULO: MORFOLOGÍA Y FISIOLOGÍA NORMAL.

El tejido testicular está compuesto por dos componentes principalmente, el tejido intersticial y los túbulos seminíferos. El tejido intersticial se conforma por células de Leydig, encargadas de la síntesis de la testosterona. Los túbulos seminíferos están integrados por células de Sertoli y espermatogonias, la cuales se diferencian durante la espermatogénesis a espermatocitos primario, secundario, espermátida, hasta la formación de espermatozoides.

Las espermatogonias son las células precursoras de los espermatozoides, tienen una alta tasa de proliferación durante la espermatogénesis (Phillips, Gassei, & Orwig, 2010; Yoshida, 2008). Existen dos tipos de espermatogonias: las A y las B. Las espermatogonias A se clasifican en dos tipos Ad y Ap. El tipo Ad (Dark), son espermatogonias de reserva, sólo se dividen por mitosis cuando las espermatogonias disminuyen drásticamente por agentes químicos o físicos genotóxicos; por ejemplo, fármacos quimioterapéuticos, agentes alquilantes, compuestos organofosforados (Insecticidas); o agentes físicos como radiaciones. Las espermatogonias Ap (Pale), por tener la característica nuclear pálido, realizan divisiones mitóticas activas, generando tanto espermatogonias de tipo Ap como tipo B. Las espermatogonias tipo B, entran al proceso de diferenciación para la producción de espermatozoides, mediante la formación gradual de diversos tipos celulares, tales como los espermatocitos primarios, espermatocitos secundarios, espermátidas y espermatozoides durante la espermatogénesis (Ahmed & de Rooij, 2009; de Rooij, 2001; Hacker-Klom, 1995; Yoshida, 2008).

ESPERMATOGÉNESIS

La espermatogénesis es el mecanismo encargado de la producción de los espermatozoides, proceso que se realiza en los túbulos seminíferos de los testículos. En el humano la espermatogénesis se inicia en la pubertad; en el ratón se produce entre el día 34-38, o incluso al día 30 después del nacimiento (durante la 4 semana), dependiendo de la cepa (Oakberg, 1956a, 1956b).

La espermatogénesis consta básicamente de cuatro fases:

1. Fase de proliferación de células germinales: Presenta una alta tasa de divisiones mitóticas de las espermatogonias Ap (2n) para generar espermatogonias tipo Ap (2n) y tipo B (2n).
2. Crecimiento: Las espermatogonias tipo B aumentan de tamaño para formar los espermatocitos primarios (2n), que inician la diferenciación celular y la migración hacia el lumen.
3. Maduración: El espermatocito primario inicia la primera división meiótica, generando dos espermatocitos secundarios (n). Posteriormente, los espermatocitos secundarios tienen la segunda división meiótica para generar 4 espermátidas (n).
4. Espermiogénesis, las espermátidas se diferencian a espermatozoides, mediante la compactación del ADN, la condensación del núcleo, reducción del citoplasma, formación del acrosoma y flagelo.

La hormona estimuladora del folículo (FSH, del inglés Follicle Stimulating Hormone), es una hormona peptídica del tipo gonadotropina, que se sintetiza y secreta en la adenohipófisis, la cual ejerce una señalización endocrina sobre el tejido testicular actuando en la síntesis del factor de células troncales (SCF del inglés, Stem Cell Factor), un ligando que se une con el receptor CKIT de las espermatogonias (Figura C). La interacción paracrina de SCF con CKIT aumenta la actividad de las vías de señalización de proliferación canónicas MAPK y PI3K/AKT (Cooke & Saunders, 2002).

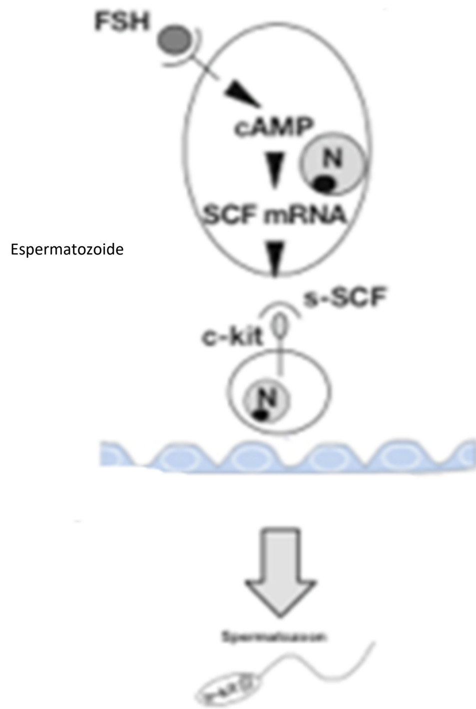

Figura C. Vía de señalización de la hormona folículo estimulante sobre la proliferación de las espermatogonias. La hormona folículo estimulante es liberada de la adenohipófisis, y transportada a células de Sertoli, mediante una señalización endócrina para la síntesis de SCF, por efecto del AMP cíclico, secretando el ligando SCF para unirse al receptor C-KIT localizado en la superficie de las espermatogonias y activar las vías de señalización proliferativas.

PAPEL DE C-KIT Y SCF EN LA PROLIFERACIÓN DURANTE LA ESPERMATOGÉNESIS.

El receptor tirosina cinasa C-Kit tipo III tiene un papel importante en la proliferación celular de las espermatogonias durante el inicio de la espermatogénesis (Rossi, Sette, Dolci, & Geremia, 2000; Yoshinaga et al., 1991; Zhang et al., 2013; Zhang et al., 2011). Esta proteína tiene un peso de 145 KDa y se localiza en la membrana de células pluri y multipotentes, tales como células germinales y células progenitoras del linaje linfoide y mieloide (Schlessinger, 2000; Yarden et al., 1987; Yuzawa et al., 2007). En el testículo el receptor C-Kit se expresa en espermatogonias (Natali et al., 1992; Sandlow, Feng, Cohen, & Sandra, 1996) (Albanesi et al., 1996), (Dym et al., 1995) hámster y cuyo (von Schonfeldt, Krishnamurthy, Foppiani, & Schlatt, 1999).

La activación del receptor C-KIT es regulada por el factor de células troncales SCF (por sus siglas en inglés) durante la etapa temprana de la espermatogénesis. El factor de células troncales SCF es un ligando peptídico de 30.9 KDa., es sintetizado y secretado por las células de Sertoli, por efecto de la Hormona Folículo Estimulante (FSH) (Manova et al., 1993; Marziali, Lazzaro, & Sorrentino, 1993; Rossi, Albanesi, Grimaldi, & Geremia, 1991; Rossi et al., 1993; Rossi et al., 2000; Tajima, Onoue, Kitamura, & Nishimune, 1991). Las células de Sertoli están localizadas en el interior de los túbulos seminíferos, tienen dos funciones: la primera es estructural y soporte a los componentes células del túbulo seminífero; la segunda es la señalización paracrina sobre el receptor de membrana C-Kit de las espermatogonias para promover la proliferación celular e iniciar la espermatogénesis, mediante la señalización de cinasas ERKs y AKT, activadas por señales extracelulares, Figura D (Dolci, Pellegrini, Di Agostino, Geremia, & Rossi, 2001; Rossi et al., 2000).

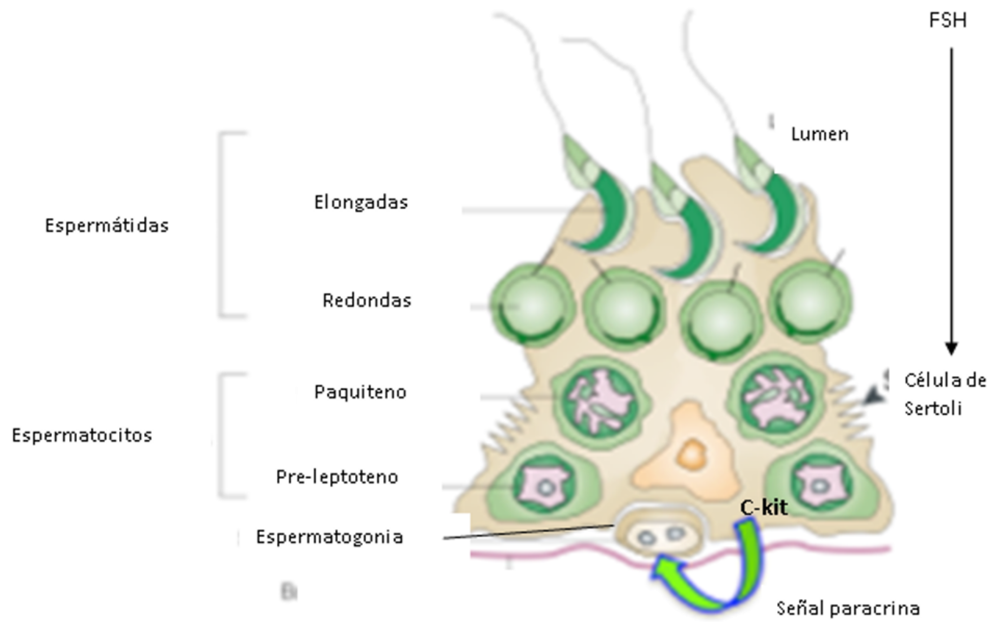

Figura D. Señal paracrina de CKIT-SCF. A) Secreción de SCF por las células de Sertoli sobre las espermatogonias. B) FSH activa la síntesis y secreción de SCF en las células de Sertoli para la activación de la señal paracrina SCF-SCF en la proliferación de espermatogonias.

VÍAS DE SEÑALIZACIÓN MAPK Y PI3K/AKT INVOLUCRADAS EN LA PROLIFERACIÓN DE LAS ESPERMATOGONIAS.

La activación del receptor C-Kit por el factor de células troncales, regula las vías moleculares de las cinasas activadas por mitogenos (MAPK) y de cinasa AKT/PI3K (fosfatidil inositol 3 Cinasa) que participan en la proliferación de espermatogonias e inhibición de la apoptosis (Dolci et al., 2001; He, Kokkinaki, Jiang, Dobrinski, & Dym, 2010).

En cultivos de espermatogonias de ratones, se demostró que la adición de SCF, factor de células troncales, incrementa las proteínas de Ciclina A y E, promueve la hiperfosforilación de la proteína de retinoblastoma Rb, acelera la transición de G1 a S del ciclo celular e incrementó la incorporación de timidina tritiada en las espermatogonias y promueve la proliferación, mediante las fosforilaciones de la cinasa ERK y AKT en los sitios Tirosina 204 y Serina 473 respectivamente (pERK ^{Y204}, pAKT ^{S473}). Ambas vías de señalización activan los siguientes complejos: Ciclina D3-CDK4, involucrado en liberación del Factor de transcripción E2F mediante la hiperfosforilación de la proteína Rb, para la síntesis de los complejos: Ciclina E-CDK2 (transición de G1/S) y Ciclina A2-CDK2 complejo involucrado en la progresión de la fase S de las espermatogonias durante la fase proliferativa de la espermatogénesis (Figura E; tomada y modificada de Dolci et al., 2001).

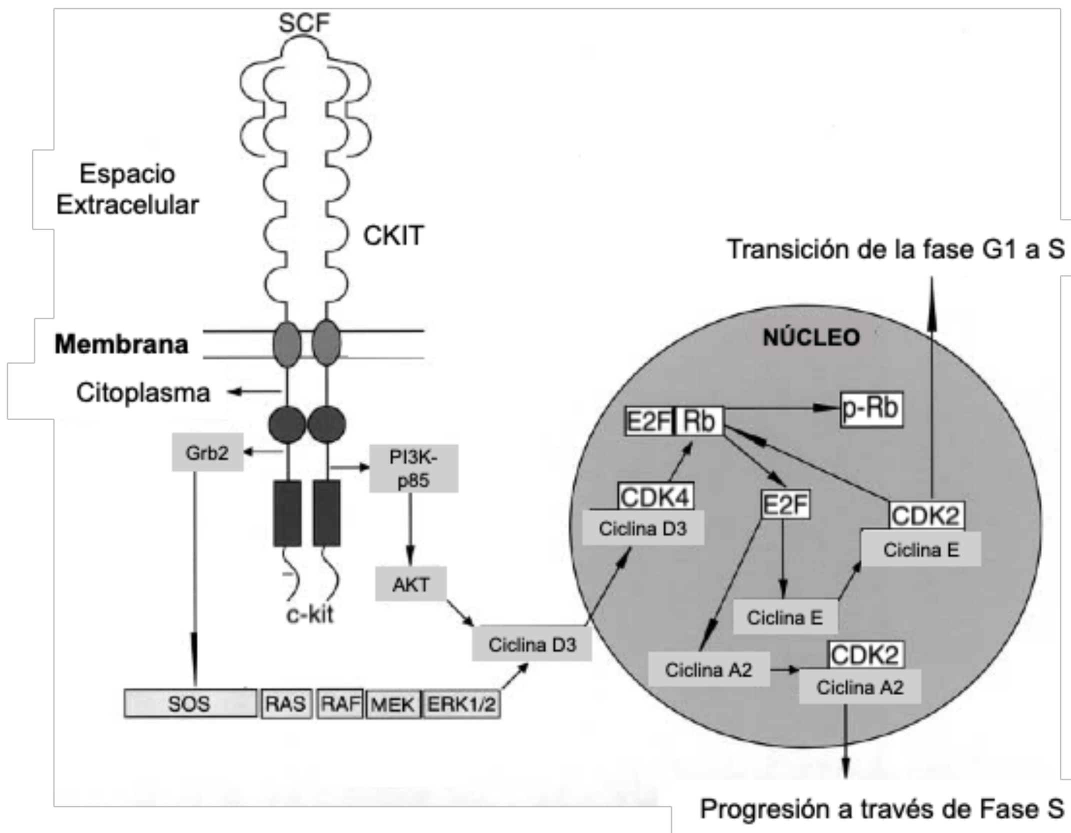

Figura E. Vías de señalización canónicas MAPK y PI3K/AKT en la proliferación de espermatogonias que convergen en la activación de Ciclina D3 para la regulación de complejos ciclinas/cinasas en la transición de las fases G1/S y la progresión durante la fase de síntesis en el ciclo celular de las espermatogonias.

COMPLEJOS CICLINA-CINASA EN LA PROLIFERACIÓN DURANTE LA ESPERMATOGÉNESIS.

La ciclina A2 tiene un papel importante en las etapas tempranas de la espermatogénesis, principalmente en la mitosis de las espermatogonias para la formación de espermatogonias tipo A y B (Figura F). La ciclina A2 es responsable de la regulación de las transiciones G1 S y G2 M, mediante la unión con su cinasa dependiente de ciclina CDK2 (Hochegger, Takeda, & Hunt, 2008; Yam, Fung, & Poon, 2002). Ciclina D participa en la regulación del ciclo celular mediante la formación de un complejo con CDK4/6 encargado de fosforilar moléculas de transición G1/S Figura F.

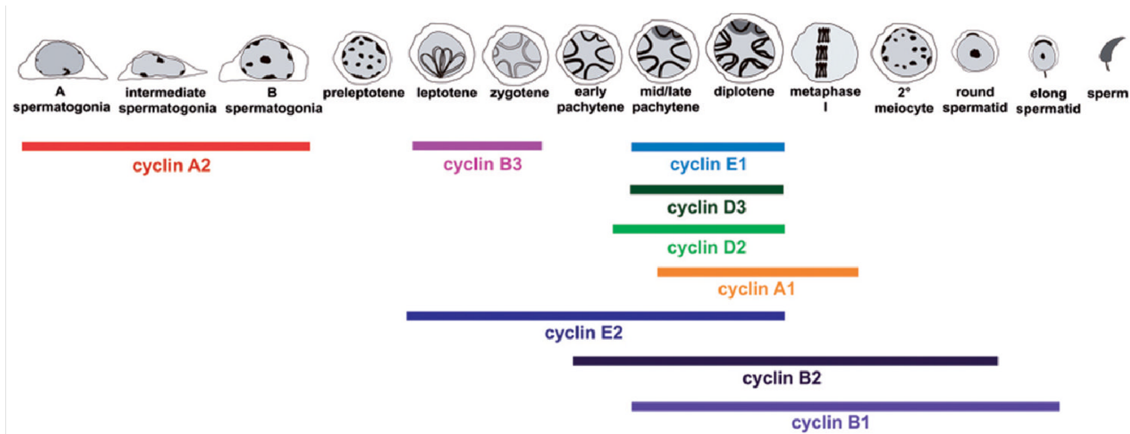

Figura F. Expresión de ciclinas durante la espermatogénesis de mamíferos. Barras coloreadas indican expresión de cada ciclina en diferentes tipos celulares durante la espermatogénesis, esta actividad ha sido reportada tanto a nivel transcripcional como traduccional (Wolgemuth, Manterola, & Vasileva, 2013).

PLANTEAMIENTO DEL PROBLEMA

Existe controversia sobre el efecto de concentraciones farmacológicas de biotina sobre la proliferación celular. Estas discrepancias podrían deberse a los diferentes modelos celulares y dosis analizadas. Estudios previos del laboratorio se encontró que en ratones Balb/cAnN, la administración de una dieta suplementada con biotina conteniendo 97.7 mg de biotina por kilogramo de dieta, aumentó el número de espermatogonias en los túbulos seminíferos. No existen estudios del efecto de concentraciones farmacológicas de biotina sobre los mecanismos moleculares de proliferación. En este trabajo proponemos estudiar, en el mismo modelo experimental en el que se han encontrado el aumento en el número de espermatogonias, cambios en las vías de señalización que participan en la proliferación de estas células.

HIPÓTESIS

La suplementación con biotina en la dieta podría estimular la proliferación de las espermatogonias, mediante moléculas de señalización endócrina y parácrina encargadas de promover el ciclo celular a través de las vías de señalización MAPK y PI3K/AKT.

OBJETIVO GENERAL

Analizar el efecto de una dieta suplementada con biotina sobre la abundancia de moléculas reguladoras de la proliferación celular en tejido testicular de ratón.

OBJETIVOS PARTICULARES

1. Medir los niveles circulantes de la hormona folículo estimulante en suero.
2. Medir la abundancia de las proteínas del factor de células troncales SCF y el receptor CKIT en tejido testicular.
3. Medir la abundancia de la proteína CKIT en espermatogonias.
4. Cuantificar los transcritos de genes reguladores del ciclo celular en tejido testicular.
5. Medir la abundancia de proteínas que participan en las vías de señalización de proliferación canónicas MAPK y PI3K/AKT en tejido testicular de ratones suplementados con una dieta farmacológica de biotina.

MATERIALES Y MÉTODOS

Modelo experimental. Se utilizó el modelo experimental de ratones en los cuales se observaron cambios en la morfología del testículo y aumento en el número de espermatogonias. Este modelo experimental se ha usado en el laboratorio ya que no ha mostrado efectos tóxicos sistémicos, ni en marcadores de toxicidad, pero se obtienen efectos hipoglucemiantes e hipolipemiantes (Larrieta *et al*., 2010; Aguilera-Méndez 2012) semejantes a los observados en humanos con dosis de farmacológicas de biotina (Coggeshall *et al.,* 1985; Maebashi *et al.,* 1993; Koutsikos *et al.,* 1996, Singer *et al.,* 2006) que son comercializadas como coadyuvantes en el tratamiento de diabetes y síndrome metabólico; además de alcanzar concentraciones séricas de biotina (Lazo-de la Vega *et al*., 2013) en el orden de magnitud similar a las que se encuentran en el ser humano con los suplementos comerciales de la vitamina (Mock 1997).

PROCEDIMIENTO.

Ratones macho de la cepa BalbC/AnN recién destetados de tres semanas de edad que no han probado alimento sólido, se dividieron en 2 grupos, con 20 ratones cada uno o 10 ratones en cada uno, los cuales se alojaron en cajas de policarbonato modelo N10-PC (Ancare corp. Bellmore, NY, USA) con 5 ratones por caja. El grupo control recibió una dieta con 1.76 mg de biotina / kg de alimento, que contiene los requerimientos diarios de la vitamina (TD 97126, Harlan Teklad, Madison WI, USA) y un grupo suplementado a la que se le administró una dieta con 97.7 mg de biotina/ Kg de alimento (TD 02453, Harlan Teklad, Madison WI, USA), cantidad que representa 56 veces el contenido de la vitamina en la dieta control. Los animales se mantuvieron en ciclos de luz/ obscuridad de 12 horas cada uno, a una temperatura promedio de 22° C, con agua y alimento *ad libitum* durante 8 semanas, A las 8 semanas de administración de las dietas se les retiró el alimento las 8 de la noche y a las 8 de la mañana los ratones se anestesiaron por inhalación con Sevoflurano (Sevorane) en una campana de flujo laminar horizontal. Se corroboró la eficacia de la anestesia por la ausencia de retracción de la pata trasera en respuesta a la punción de la almohadilla dactilar del pie, la ausencia del reflejo cornal y el reflejo de pinzamiento de la cola.

AISLAMIENTO DE ESPERMATOGONIAS

La disociación de las espermatogonias se realizó mediante la digestión con colagenasa tipo-1 y tripsina siguiendo el protocolo (Liu et al., 2011). Brevemente, un testículo de cada ratón se cortó en trozos pequeños y se lavaron dos veces con la solución de Hank’s conteniendo penicilina 200 U/ml y estreptomicina 200 mg/ml. El tejido se digirió a temperatura ambiente con 1 g/L de colagenasa tipo-I con agitación moderada por 5 min, posteriormente se dejó sin agitación por 5 minutos adicionales y se centrifugó a 4,500 g por 5 min. El sobrenandente se removió y el pelet restante se trató con una solución al 0.25% de tripsina a 37°C por 5 min con agitación suave. Las células disociadas se centrifugaron y filtraron en una malla de nylon. Las células que se recuperaron se suspendieron en medio de cultivo DMEM (Life Technologies, Carlsbad, CA, USA) conteniendo 10% de suero bovino fetal (Sigma-Aldrich, St. Louis, MO, USA). La suspensión celular se agregó a un gradiente de Percoll gradiente al 11%, 19%, 27%, 35%, y 43% distribuidas de la más alta a la más baja concentración, centrifugándose posteriormente a 353 g por 30 min. Las células situadas en el gradiente de Percoll en las concentraciones de 27% to and 35%, las cuales corresponden a las espermatogonias, se recolectaron, lavaron dos veces con PBS, y se resuspendieron en PBS ajustando a una densidad celular de 1 × 105/ml. Un volumen de 200 µL de la suspensión de espermatogonias de cada muestra se lisó con la solución RIPA (Thermo Scientific, St. Peters, MO, USA) conteniendo 1% de inhibidores de proteasas (Roche, Mannheim, Germany). Los lisados se centrifugaron a 12,500 g, por 15 min at 4°C. Un volumen de 30µl del sobrenadante conteniendo 2.5 x 104células de cada muestra se analizó por electroforesis con geles de sodio dodecil sulfato-poliacrilamida (SDS-PAGE) al 6%.

ANÁLISIS DE LA HORMONA FOLÍCULO ESTIMULANTE (FSH).

La sangre se obtuvo de la vena cava posterior y se dejó coagular por 30 minutos a 20°C en tubos Eppendorf estériles de 1.5 ml (Axigen, Union City, CA, USA). El coágulo se removió por centrifugación a 500 x g por 10 min at 4°C y el sobrenadante resultante se removió con una pipeta Pasteur a tubos Eppendorf estériles de 1.5 ml. El suero se almacenó a −20°C. La hormona folículo estimulante se determinó por ELISA usando un kit comercial de acuerdo con las instrucciones del manufacturador (MBS703380 ELISA kit, MyBioSource, San Diego, CA; U.S.A). El rango de detección del kit es 4 mIU/ml- 140 mIU/ml. El límite de detección del kit es 2.5mIU/ml. Coeficiente de variación: intraensayo <15%; interensayo <15%. Las muestras se analizaron por duplicado. Todos los valores obtenidos estuvieron en el rango de detección del kit.

AISLAMIENTO DEL ARN Y ANÁLISIS DE TRANSCRITOS POR RT-PCR EN TIEMPO REAL

El ARN total se aisló de uno de los testículos usando el reactivo de TRIzol Reagent siguiendo las instrucciones de la compañía productora (Invitrogen, Carlsbad, CA, USA) y como fue descrito previamente (Moreno-Méndez et al., 2019). La calidad del ARN se analizó por electroforesis en un gel de agarosa y por espectrofotometría determinando la relación de absorbancia medida a 260/280 de longitud de onda (BioTek, Winooski, VT, USA). Todas las muestras presentaron relaciones de densidad óptica entre 1.9–2.1. El cDNA se sintetizó a partir de 1 µg de ARN total mediante la reacción de transcripción reversa con 500 unidades de M-MVL RT (Invitrogene, Carlsbad, CA, USA) y hexámeros random (Invitrogene, Carlsbad, CA, USA). El cDNA se mezcló con Sybr Green Máster mix (Applied Biosystems, Woolston, Warrington, UK) y la secuencia pertinente de primers específicos los cuales se describen en la **Tabla 1.** La deteminación de la abundancia de los transcritos se realizó por PCR en tiempo real usando el aparato ABI Prism 7700 Sequence Detector (Foster City, CA, USA). La expresión cuantitativa de los genes se calculó por el método de doble CT ($2^{-\Delta\Delta CT}$) descrito por Livak y Schmittgen (Livak and Schmittgen, 2001). Las muestras se analizaron por triplicado. Como control interno se usó la cuantificación de tubulina. abundancia en la expresión de los genes se calculó el valor umbral del ciclo (CT, cyclethreshold) usando el método de cuantificación relativa doble-delta CT ($2^{-\Delta\Delta CT}$). Todas las muestras se analizaron por triplicado y la media de la expresión de cada gen del tratamiento suplementado fue normalizado respecto a la media de la expresión del tratamiento control como se describió previamente (Moreno-Méndez et al., 2019).

ANÁLISIS DE WESTERN BLOT

Un testículo de cada ratón se homogenizó con un polytron (Kinematica AG-Polytron PT 2100, Luzern, Switzerland) en una solución amortiguadora conteniendo 50 mM HEPES, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM ortovanadato, 25 mM fluoruro de sodio, 5 mM de pirofosfato de sodio, 1 mM ditiot0reitol, 0.1% TritonX-100 (Sigma, St. Louis, MO, USA) e inhibidores de proteasas (Roche, Mannheim, Germany). La suspensión se centrifugó a 12,500 x g por 30 min at 4ºC. La proteína obtenida se cuantificó por espectrofotometría usando una solución de Bradford (Bio-Rad, Richmond, CA, USA) usando como patrón una solución de albúmina de suero bovina. Un volumen de 30 µg de proteína se puso a ebullición en solución de Laemmli (Bio-Rad, Richmond, CA; USA) por 5 min y posteriormente se sometió a electroforesis en geles de (SDS-PAGE). Para la determinación de c-kit se usaron geles al 6% de poliacrilamida, para la determinación de ERK1/2p^{Tyr204}, ERK1, ERK2, AKT^{Ser473}, AKT^{Thr308}, AKT y GAPDH, se usaron geles al 10% y del 15% para Ciclina D3 y ERK1. Las proteínas se transfirieron a membranas de nitrocelulosa (Bio-Rad, Hercules, CA, USA) como descrito previamente (Aguilera-Méndez and Fernández-Mejía, 2012) e identificado con los anticuerpos correspondientes descritos en la tabla (**Table 2**).

ANÁLISIS ESTADÍSTICO

Los resultados se expresan como el promedio $\pm$ error estándar. La evaluación estadística se realizó con la prueba de t-de Student. El programa GraphPad Prisma 6.0 (Berkeley, CA, USA) se usó para el análisis estadístico. La significancia estadística se consideró significativa con valores de P menores a 0.05.

RESULTADOS

EFECTO DE LA DIETA SUPLEMENTADA CON BIOTINA DURANTE 8 SEMANAS SOBRE LAS CONCENTRACIONES SÉRICAS DE LA HORMONA FSH Y EL FACTOR SCF.

Se determinaron las concentraciones séricas de la hormona folículo estimulante con el fin de evaluar si el aumento de la proliferación de las espermatogonias observada en estudios previos (Pastén-Hidalgo et al., 2020) se encuentra relacionada con efectos de la biotina sobre la secreción de esta hormona. Como se ilustra en la **Figura 1A**, no se encontró diferencia entre los grupos en cuanto a la concentración sérica de esta hormona (dieta control: 8.55 ± 0.23; dieta suplementada con biotina: 8.52 ± 0.16 mIU/mL; P=0.90). El factor de células troncales (SCF) se produce y secreta en el tejido testicular en respuesta a la hormona folículo estimulante (Rossi et al., 2000). De acuerdo con la falta de efecto de la dieta suplementada con biotina sobre las concentraciones séricas de esta hormona, nuestros datos revelaron que la expresión de esta proteína es similar entre el grupo control y el experimental (dieta control: 1.21 ± 0.06; dieta suplementada con biotina: 1.28 ± 0.070; P=0.46) (**Fig. 1B**)

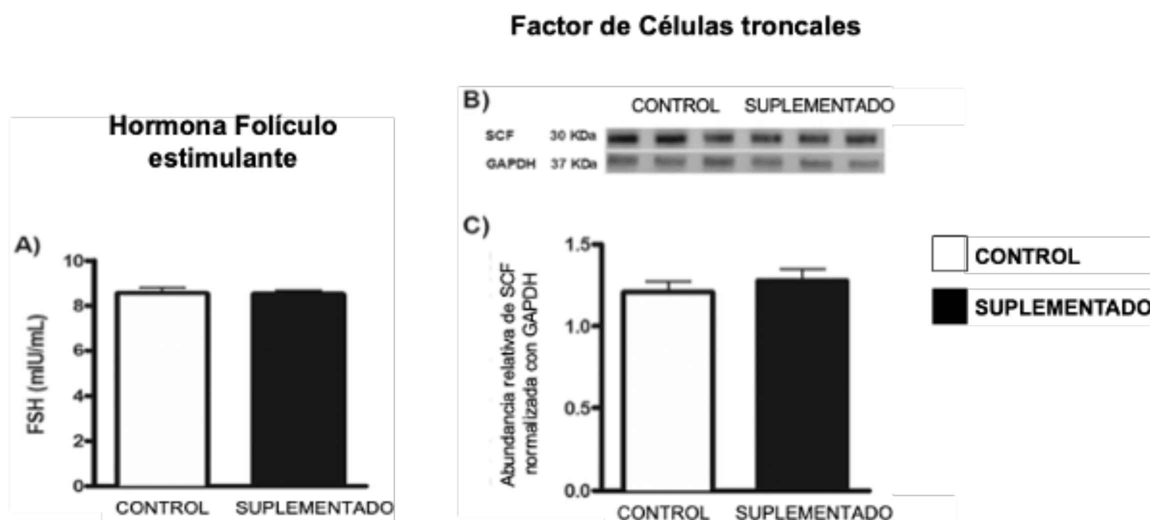

Figura 1. Efecto de una dieta suplementada con biotina sobre las concentraciones séricas de la hormona folículo estimulante. A) Cuantificación de las concentraciones séricas de la hormona folículo estimulante Las barras representan el promedio ± ES de 16 ratones por grupo. **B)** Western Blot representativo de la abundancia del análisis del factor de células troncales y de la enzima gliceraldehído fosfato deshidrogenasa. **C)** Cuantificación del factor de células troncales normalizado con gliceraldehído fosfato deshidrogenasa. Las barras representan el promedio ± ES de 8 ratones de cada grupo.

EFECTO DE LA DIETA SUPLEMENTADA CON BIOTINA DURANTE 8 SEMANAS SOBRE LA EXPRESIÓN DE TRANSCRITOS REGULADORES DEL CICLO CELULAR.

Los datos ilustrados en la Figura 2, revelaron que, comparado el grupo control, la administración de la dieta suplementada con biotina mostró aumentos en abundancia del ARNm la ciclina D3 (inducción de 0.68 ± 0.17 veces; P=0.003) y *Cdk4* (0.55 ± 0.18 veces; P=0.011); de igual manera la expresión del mensajero de *Ciclina E* se incrementó (0.32 ± 0.11 veces; P=0.030) y del transcrito *Cdk2* se indujo positivamente 0.58 ± 0.21 veces; P=0.016). La expresión de los transcrito de la *Ciclina A2* aumentó aproximadamente el doble (0.94 ± 0.037 veces; P=0.021) y un aumento aún mayor se observó en la expresión del mensajero del factor de transcripción *E2F*, proteína de acción positiva para la progresión del ciclo celular (1.81 ± 0.80 (P=0.041). La determinación del mensajero del inhibidor *p21* mostró una disminución de éste; sin embargo, el inhibidor *p16* no presentó valor significativo para determinar aumento 2.99 ± 1.42 veces (P= 0.051).

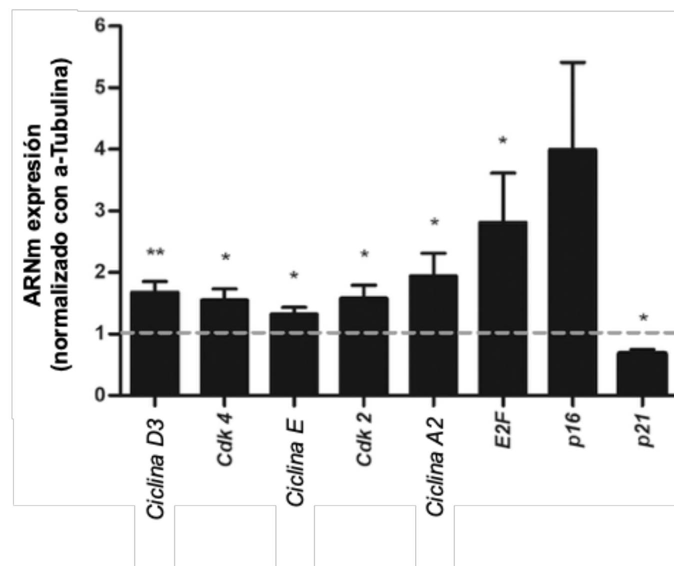

Figura 2. Efecto de una dieta suplementada con biotina sobre la abundancia del ARNm de genes del ciclo celular. Cuantificación de *Ciclina D3*, *Cdk4*, *Ciclina E*, *Cdk2*, *Ciclina A2*, *E2F*, *p16*, y *p21*. Normalizado contra alfa-tubulina. Los niveles de expresión se ven como las veces de cambio con respecto a la expresión del control (línea punteada) Las barras representan el promedio ± ES de 10-12 ratones por grupo. *P ≤ 0.05; **P ≤ 0.0005.

EFECTO DE LA DIETA SUPLEMENTADA CON BIOTINA DURANTE 8 SEMANAS SOBRE LA EXPRESIÓN DE TRANSCRITOS DE LOS FACTORES TRANSCRIPCIONALES SP1 Y SP3.

También investigamos la expresión del ARNm de los factores transcripcionales *Sp1* y *Sp3* proteínas asociadas con la proliferación celular (Safe et al., 2014), cuya expresión se ha reportado se encuentra regulada por concentraciones farmacológicas de biotina (Griffin et al., 2003). Como se muestra en la **Figura 3**, comparada con la expresión del grupo control, los transcritos de *Sp1* y *Sp*3 se vieron aumentados: *Sp1* (0.70 ± 0.19 veces; P=0.004) y *Sp3* (1.67 ± 0.28 veces; P=0.0001).

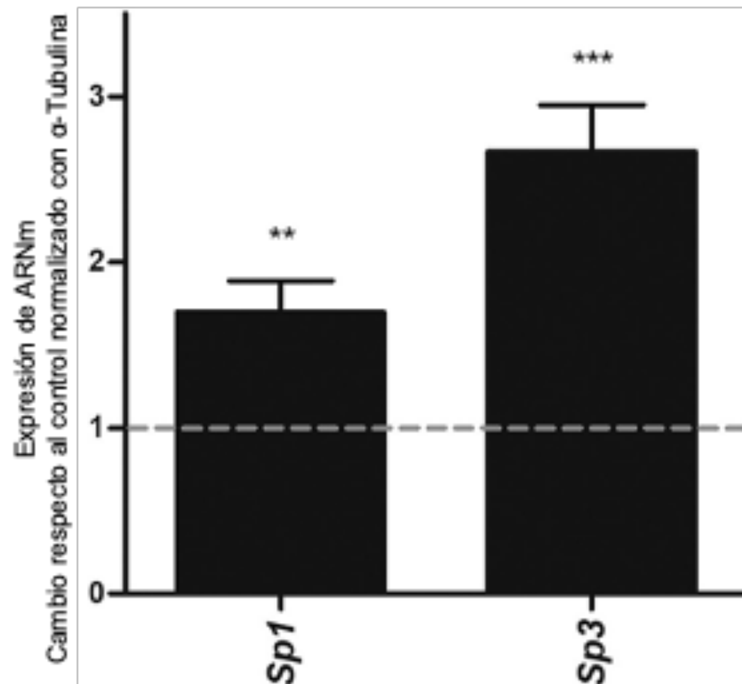

Figura 3. Efecto de una dieta suplementada con biotina sobre la abundancia del RNAm de los factores transcripcionales *Sp1* y *Sp3*. Cuantificación los factores transcripcionales *Sp1* y *Sp3* normalizado contra alfa-tubulina. Los niveles de expresión se expresan como veces de cambio con respecto a la expresión del control (línea punteada) Las barras representan el promedio ± ES de 10-12 ratones por grupo. *P ≤ 0.05; **P ≤ 0.0005.

EFECTO DE LA DIETA SUPLEMENTADA CON BIOTINA DURANTE 8 SEMANAS SOBRE LA ABUNDANCIA DE PROTEÍNAS DE LA VÍA DE SEÑALIZACIÓN MAPK Y PI3K/AKT.

Las señales provenientes de las vías de transducción de señales MAPK y PI3K/AKT convergen en la activación de la Ciclina D3 (Bayascas and Alessi, 2005; Braydich-Stolle et al., 2007; Dolci et al., 2001; Feng et al., 2000; Fu et al., 2018; Rossi et al., 2000; Wei et al., 2018). Por lo que investigamos la participación de estas proteínas en la inducción de la proliferación de las espermatogonias. Como se muestra en la **Figura 4**, se observó un aumento en la abundancia de la proteína Ciclina D3 (dieta control: 3.14 ± 0.41; dieta suplementada con biotina: 4.62 ± 0.42; P=0.030). Las proteínas que convergen en la activación de esta ciclina: la forma activa de ERK y AKT^{Ser473}, **Figura 5** se vieron aumentada en el tejido testicular de los ratones alimentados con la dieta rica en biotina: $pERK1/2^{Tyr204}$ (dieta control: 1.14 ± 0.19; dieta suplementada con biotina: 2.14 ± 0.23; P=0.005); $pAKT^{Ser473}$ (dieta control: 0.62 ± 0.31; dieta suplementada con biotina: 1.95 ± 0.22; P=0.001). La abundancia de la forma total de ERK2 también aumentó (dieta control: 1.04 ± 0.025; dieta suplementada con biotina: 1.47 ± 0.16; P=0.003), no así la abundancia de las proteínas ERK1, AKT, and $pAKT^{Thr308}$.

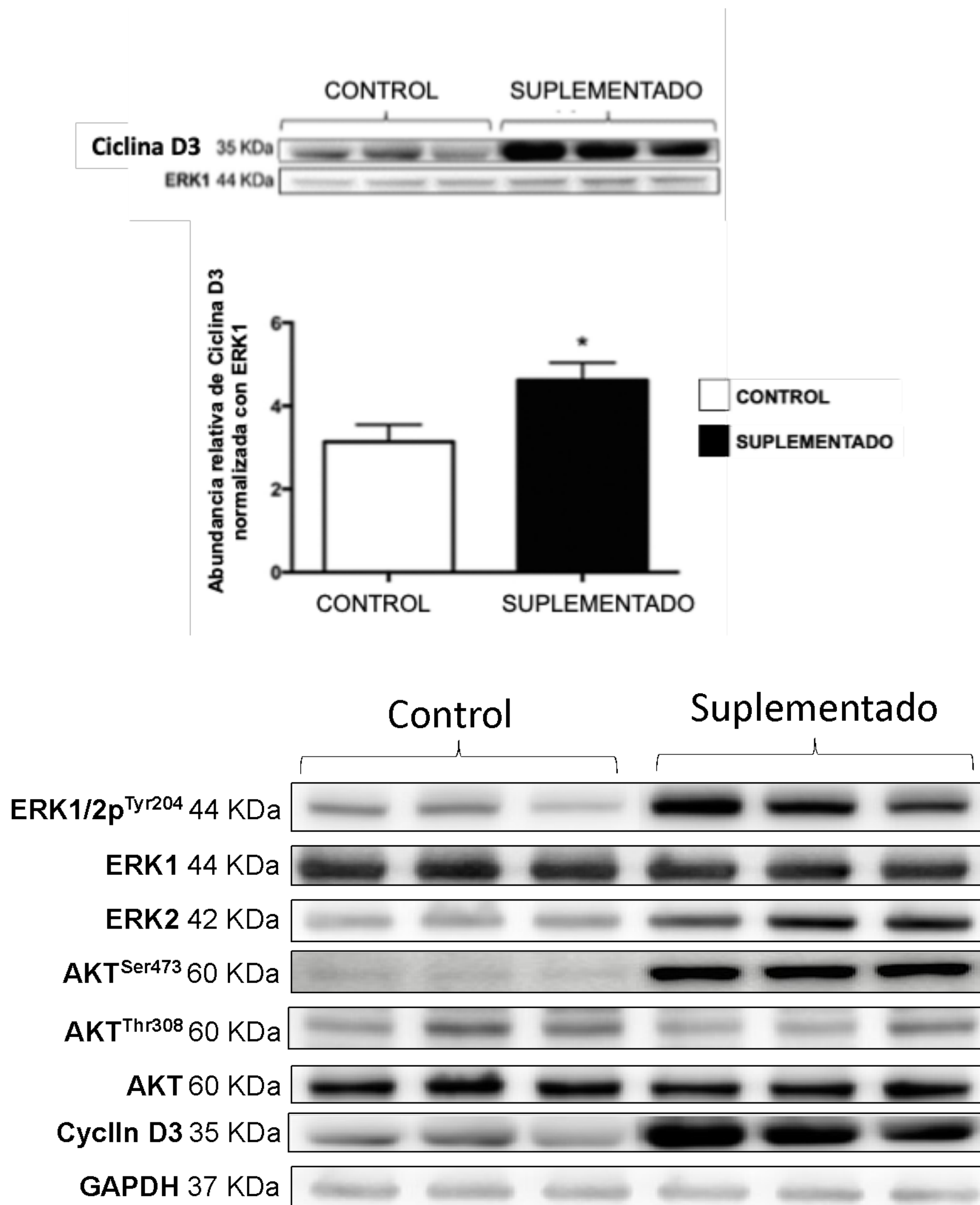

Figura 4. Efecto de una dieta suplementada con biotina sobre la abundancia proteica de la ciclina D3. Panel superior: Western Blot representativo de la abundancia proteica de ciclina D3, normalizado con ERK1. **Panel inferior.** Barra blanca: grupo control; barra negra: grupo suplementado con biotina. Las barras representan el promedio ± ES de 6 ratones por grupo. *$P \leq 0.05$

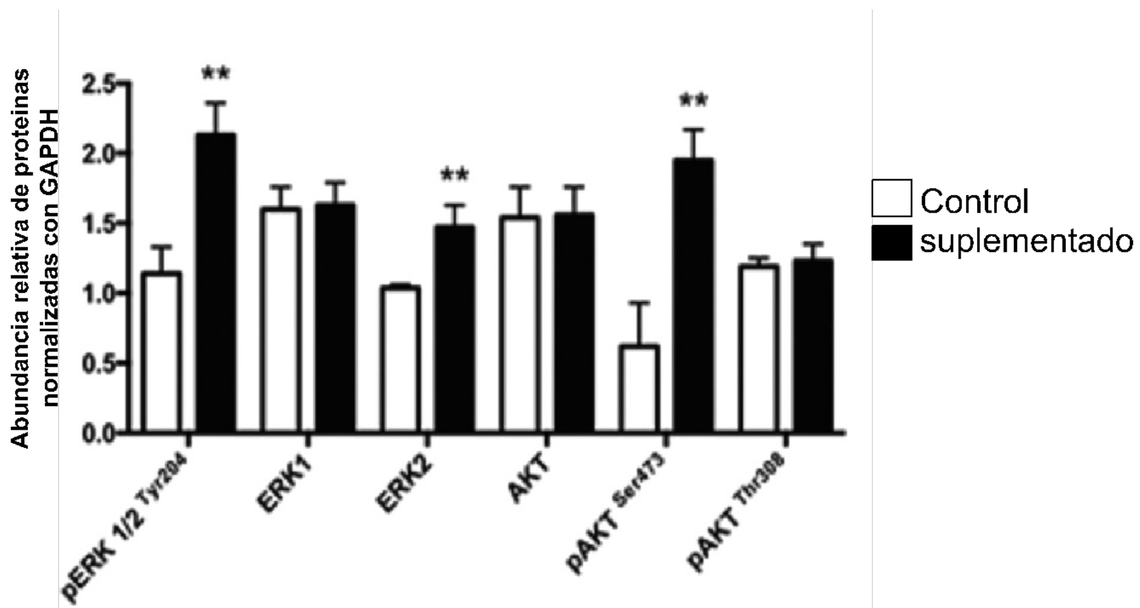

Figura 5. Efecto de una dieta suplementada con biotina sobre la abundancia de proteínas de la vía de transducción de señales MAPK y ERK. Panel superior: Western blot representativo de la abundancia proteica de pERK1/2^{Tyr204}, total ERK1, ERK2 and AKT, pAKT^{Ser473}, AKT^{Thr308}, normalizado con gliceraldehído-3 fosfato deshidrogenasa. **Panel inferior.** Barras blancas: grupo control; barras negras: grupo suplementado con biotina. Las barras representan el promedio ± ES de 8 ratones por grupo. $^*P \leq 0.05$; $^{**}P \leq 0.005$.

EFECTO DE LA DIETA SUPLEMENTADA CON BIOTINA DURANTE 8 SEMANAS SOBRE LA ABUNDANCIA DEL RECEPTOR C-KIT.

Las vías de señalización MAPK y PI3K/AKT se activan por la interacción entre el receptor c-kit-receptor y su ligando el factor de células troncales SCF (Dolci et al., 2001; Feng et al., 2000; Rossi et al., 2000). Analizamos si el efecto de la dieta suplementada con biotina está relacionado con el aumento del receptor c-kit. Como se muestra en las **Figuras 6A y B**, se produjo un aumento en la expresión testicular de la proteína del receptor c-kit- (dieta control: 0.86 ± 0.16; dieta suplementada con biotina: 2.03 ± 0.38; P=0.012). Dado que en estudios previos (Pastén-Hidalgo et al., 2020) encontramos que ocho semanas de la ingesta de esta dieta aumenta el número de espermatogonias, comparamos la expresión de c-kit en muestras proteicas conteniendo igual número de espermatogonias. Los datos revelaron que la abundancia de c-kit se vio incrementado (dieta control: 0.86 ± 0.13; dieta suplementada con biotina: 1.06 ± 0.05); P=0.038) **Figuras 6C y D**.

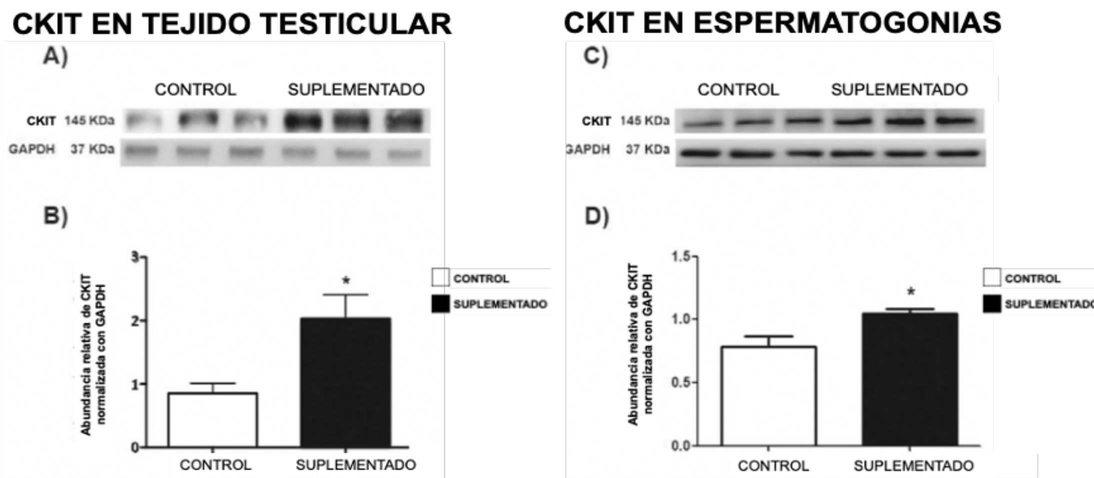

Figura 6. Efecto de una dieta suplementada con biotina sobre la abundancia de c-kit y GAPDH. Panel C: Western Blot representativo de la abundancia proteica de c-kit y GAPDH en el testículo; **Panel A.** Western Blot representativo de la abundancia proteica de c-kit y GAPDH en espermatogonias aisladas. **Panel D**. Cuantificación del receptor c-kit normalizado con GADPDH en homogenados de testículo. Las barras representan el promedio ± ES de 8 ratones por grupo. *P < 0.05. **Panel B.** Cuantificación del receptor c-kit normalizado con GADPDH en extractos de espermatogonias aisladas. Barras blancas: grupo control; barras negras: grupo suplementado con biotina. Las barras representan el promedio ± ES de 5 ratones por grupo. *P < 0.05.

DISCUSIÓN.

Ha sido bien documentado que las concentraciones farmacológicas de biotina tienen efectos independientes de su acción como grupo prostético de las carboxilasas, los cuales se producen por mecanismos en la expresión de genes al nivel transcripcional, traduccional y post-traduccional (Mock, 2017; Riverón-Negrete and Fernandez-Mejia, 2017; Zempleni et al., 2009).

Debido a que varios de los efectos que produce la biotina en concentraciones farmacológica tienen potencial terapéutico en diversas afecciones como la diabetes, el síndrome metabólico, la esclerosis múltiple, entre otros (Mock, 2017; Riverón-Negrete and Fernandez-Mejia, 2017), diversos estudios se han enfocado en la identificación de los mecanismos moleculares mediante los cuales la vitamina produce esos efectos.

Existe evidencia de que concentraciones farmacológicas de biotina modifica la proliferación celular (Crisp et al., 2004; Griffin et al., 2003; Manthey et al., 2002; Valenciano et al., 2002; Zempleni et al., 2001); sin embargo, es poca la información sobre los mecanismos de esta vitamina sobre la proliferación. En estudios recientes encontramos que ocho semanas de una dieta suplementada 56 veces la cantidad de biotina presente en la dieta control aumentó el número de espermatogonias en los túbulos seminíferos y la expresión del marcador de proliferación Ki67 en estas células (Pastén-Hidalgo et al., 2020). En este estudio analizamos los mecanismos moleculares que se encuentran asociados con los efectos de la suplementación con biotina sobre la proliferación celular en el testículo de ratón.

La hormona folículo estimulante actúa como un inductor de la proliferación de las espermatogonias (Casarini and Crépieux, 2019). Nuestros estudios no encontraron diferencias significativas entre los grupos en las concentraciones séricas de la hormona, indicando que los efectos de la dieta suplementada con biotina no se producen mediante el eje hipotalámico-pituitario-testicular. En consonancia con la falta de efecto de la biotina sobre las concentraciones séricas de esta hormona, nuestros resultados encontraron que la expresión del factor de células troncales SCF, un factor que se expresa y secreta bajo la inducción de la hormona folículo estimulante (Rossi et al., 2000), no difiere entre los grupos.

A diferencia de la falta de efecto en la hormona folículo estimulante y SCF, nuestros resultados encontraron que el receptor de este factor, denominado c-kit, se encontró significativamente aumentado en el grupo que recibió la dieta suplementada con la vitamina, al igual que las formas activas de ERK ($pERK1/2^{Tyr204}$) y AKT^{Ser473}, lo que indica que el efecto de la biotina se produce sobre las vías canónicas de proliferación. Este hallazgo, amplía la información obtenida en estudios de proteómica (Rodríguez-Meléndez et al. 2005) en el cual se encontró que la biotina modificó diversas proteínas clasificadas dentro de la categoría de vías de señalización; sin embargo, la identidad de estas proteínas no fue descrita en estos estudios, por lo que esta investigación identifica por primera vez las moléculas de transducción se señales que responden a la acción de la suplementación con biotina.

Igualmente, este trabajo de tesis expande observaciones obtenidas en estudios de microarreglos realizadas en células de sangre periférica aisladas de individuos normales a los que se les administró 1.5 mg de biotina 50 veces los requerimientos diarios (Wiedmann et al., 2004), que describieron que uno de los cambios más importantes con el tratamiento se observó en los transcritos relacionados con la proliferación en el ciclo celular, encontrando que la suplementación con biotina en la dieta durante ocho semanas aumentó la expresión de la Ciclina D3, proteína que se transloca hacia el núcleo y causa la activación del ciclo celular al nivel de G1/S

durante la proliferación (Dolci et al., 2001; Feng et al., 2000; Hasegawa et al., 2013). Concordantemente con este proceso nuestros estudios encontraron aumentos de los transcritos de activadores del ciclo celular: *Ciclina D3*, *Cdk4*, *Ciclina E*, *Cdk2*, *Ciclina A2*, y el factor transcripcional *E2F* y el inhibidor *p21,* así como un intenso aumento en el ARNm del inhibidor *p16*, una proteína que previene la progresión de la fase G1/S.

Con respecto al inhibidor p16 que se encontró aumentado, se sabe que su expresión puede ser activada por especies reactivas de oxígeno, daño del ADN o senescencia, produciendo su acumulación (Kaneto et al., 1999; Qiu et al., 1996; Xin-Chang et al., 2002). Varios estudios han reportado que la suplementación con biotina no produce estrés oxidante en el hígado (Aldahmash et al., 2016; Riverón-Negrete et al., 2016; Riverón-Negrete and Fernandez-Mejia, 2017; Sahin et al., 2013), el riñón (Aldahmash et al., 2015), o el suero (Sahin et al., 2013); sin embargo, dado que el tejido testicular es muy sensible a los tóxicos, los medicamentos (Bonde, 2010; Meistrich, 1986), y posee alta capacidad para acumular a la biotina (Sawamura et al., 2015), no podemos descartar la posibilidad de que las concentraciones farmacológicas de la biotina en el testículo produzca estrés oxidante, por lo que se requerirán estudios ahondando la posibilidad de que la suplementación con biotina pudiese producir estrés oxidante en el testículo y, como consecuencia cause incremento en *p16*.

El testículo está compuesto por diferentes tipos de células: espermatogonias, células mieloides peritubulares, células de Sertoli y de Leydig. El receptor c-kit-receptor se expresa predominantemente en espermatogonias y es usado como marcador de proliferación de estas células. En estudios previos (Pastén-Hidalgo et al., 2020) demostramos que la dieta usada en la presente investigación produjo un aumento del número de espermatogonias y en su proliferación. Los resultados de esta tesis revelaron que en este mismo modelo experimental se produjo un aumento en la expresión de c-kit en los extractos proteicos de testículo completo, al igual que en los extractos de espermatogonias aisladas con igual número de estas células; sin embargo, el incremento fue mayor en la preparación de espermatogonias aisladas.

Es importante señalar que el análisis de las proteínas de la vía de señalización de MAPK y AKT se determinó en el testículo completo, el cual está compuesto de diferentes tipos celulares que expresan estas proteínas de señalización (Lai et al., 2014; Ni et al., 2019; Thuillier et al., 2009; Yamashita et al., 2011), por lo que no podemos descartar que en los aumentos observados en las proteínas de estas vías puedan estar participando las diferentes células que constituyen el testículo. Sin embargo, dado que las otras células testiculares tienen poca capacidad de proliferación (Benton et al., 1995; Orth et al., 1988) y los estudios de Pastén-Hidalgo (Pastén-Hidalgo et al., 2020) demostraron que el marcador de proliferación Ki67 se encuentra restringido en las espermatogonias, es poco probable que otras células testiculares participen en los efectos observados en este estudio sobre la expresión de las ciclinas.

El hallazgo de que el factor de células troncales SCF no se modifique en los ratones suplementados con la biotina sugiere que la vitamina directamente afecta la expresión de genes de la maquinaria de proliferación. En soporte a este punto de vista, varios estudios han encontrado que la biotina ejerce un efecto directo sobre la proliferación en cultivos celulares (Crisp et al., 2004; Griffin et al., 2003; Manthey et al., 2002) y en otros tipos de células (Pastén-Hidalgo et al., 2020; Tixi-Verdugo et al., 2017), más aún, en apoyo de una acción directa de la biotina en factores de proliferación, estudios de Griffin et al., (Griffin et al., 2003) encontraron que en la línea celular Jurkat, concentraciones farmacológicas de biotina aumentaron la expresión de los factores transcripcionales Sp1 y Sp3, proteínas asociadas con la duplicación celular (Safe et al., 2014), que se expresan en las espermatogonias (Ma et al., 2008; Thomas et al., 2005). Sp1 también regula la transcripción de c-kit (Yasuda et al., 1993). Sin embargo, no podemos descartar que otros factores de transcripción pudieran también participar en la fase proliferativa de las espermatogonias.

Existen pocas investigaciones abordando los efectos de concentraciones farmacológicas de biotina sobre el testículo (Sawamura et al., 2015, 2007). Estudios *in vivo*, realizados por Sawamura encontraron que en ratas la ingesta de una dieta conteniendo 10,000 mg de biotina/kg de alimento durante seis semanas decrementó el número de espermatogonias y produjo muerte celular por apoptosis en el epitelio espermático de los tubos seminíferos (Sawamura et al., 2015, 2007); sin embargo, esta cantidad de biotina afectó negativamente el peso corporal, la ingesta diaria de alimento y mostró ser tóxica produciendo 50% de mortalidad (Sawamura et al., 2015, 2007), por lo que es altamente probable que esta cantidad de biotina sobre el número de espermatogonias se deba un efecto tóxico generalizado. En contraste, en nuestro modelo experimental en el cual la dieta contiene 97.7 mg biotina/kg se observó un incremento en el número de espermatogonias proliferativas (Pastén-Hidalgo et al., 2020), sin modificarse el peso corporal, la ingesta de alimentos (Báez-Saldaña et al., 2009; Lazo de la Vega-Monroy et al., 2013).

La hormona folículo estimulante actúa sobre la síntesis de SCF para estimular la señalización paracrina SCF/CKIT en la activación de las vías de proliferación canónicas a partir de la segunda semana del destete. Es importante notar que estudios clásicos de mutagénesis (Hayes et al., 1984; SRI-International, 1979) han reportado que la biotina no es tóxica (Fiume, M.Z., 2021), ni afecta marcadores de estrés oxidativo o daño tisular: en el modelo experimental usado en este trabajo no se observaron efectos sobre el malondialdehido y el glutatión en el hígado, ni cambios en las enzimas de escape hepáticas. En pacientes con deficiencia genética de biotinidasa y holocarboxilasa sintetasa, dosis orales de biotina de hasta 100 mg biotina/día no se detectaron efectos tóxicos (Baumgartner and Suormala, 1997). Investigaciones clínicas en pacientes con diabetes 9 mg de biotina diaria durante 48 meses no agravó el padecimiento o produjo efectos indeseables (Maebashi et al., 1993). En pacientes con neuropatía periférica sugieren la administración de biotina de 1.5–3 mg diarios por 1–2 años para su tratamiento (Koutsikos et al., 1990). Ninguno de estos estudios analizó los efectos de la biotina sobre funciones reproductivas.

A pesar de que la suplementación con biotina en la dieta en nuestro modelo experimental ha mostrado efectos favorables en la homeostasis de la glucosa y de los lípidos (Aguilera-Méndez and Fernández-Mejía, 2012; Larrieta et al., 2010; Lazo de la Vega-Monroy et al., 2013), sin presencia de marcadores de toxicidad y efecto hepatóxico (Riverón-Negrete et al., 2016). Es imprescindible continuar la investigación sobre los efectos de la suplementación con biotina en el área de la salud reproductiva.

Los resultados en mi investigación mostraron aumento en las moléculas reguladoras del ciclo celular, así como moléculas de señalización MAPK y AKT, sin cambios en moléculas de señalización endocrina y paracrina;interesantemente, diversos estudios han asociado el aumento de activadores del ciclo celular como Ciclina A2 y Cdk2 con el desarrollo de tumores de células germinales (Liao et al., 2004). Esta evidencia en conjunto con nuestros hallazgos de la alteración en la histología

testicular, estructura y motilidad de los espermatozoides (Pastén-Hidalgo et al., 2020), alertan sobre el consumo indiscriminado y poco regulado de la suplementación con biotina en altas cantidades, la cual ha aumentado en los últimos años (John and Lipner, 2019) a pesar de que no existe evidencia suficiente de su eficacia (Soleymani, T., Lo Sicco, K., & Shapiro, 2017).

Es importante notar que los niveles de biotina que se alcanzan con complementos comerciales conteniendo (5–10 mg) son del mismo orden de magnitud de los que se encuentran en nuestro modelo experimental (Lazo de la Vega-Monroy et al., 2013; Mock and Mock, 1997).

En resumen, los resultados de este estudio encontraron que la ingesta de la dieta suplementada con biotina incrementó el receptor c-kit y las proteínas activas de las vías de señalización ERK/AKT, al igual que factores transcripcionales *Sp1* y *Sp3* y transcritos de proteínas participantes en el ciclo celular; este efecto fue independiente de la hormona folículo estimulante y del factor de células troncales SCF (Figura 8). El presente trabajo identifica por primera vez los mecanismos moleculares participantes en el efecto de concentraciones farmacológicas de biotina sobre la proliferación celular y, lo que es muy importante, genera alerta sobre el potencial hiperplásico sobre las células testiculares que podrían tener los suplementos de biotina. Estos resultados hacen apremiante la investigación para determinar los límites máximos de biotina permitidos (UL), los cuales hasta la fecha no se encuentran establecidos (Walter P. 2001).

MOLÉCULAS ESTUDIADAS EN EL MECANISMO DE PROLIFERACIÓN CELULAR DEL TEJIDO TESTICULAR.

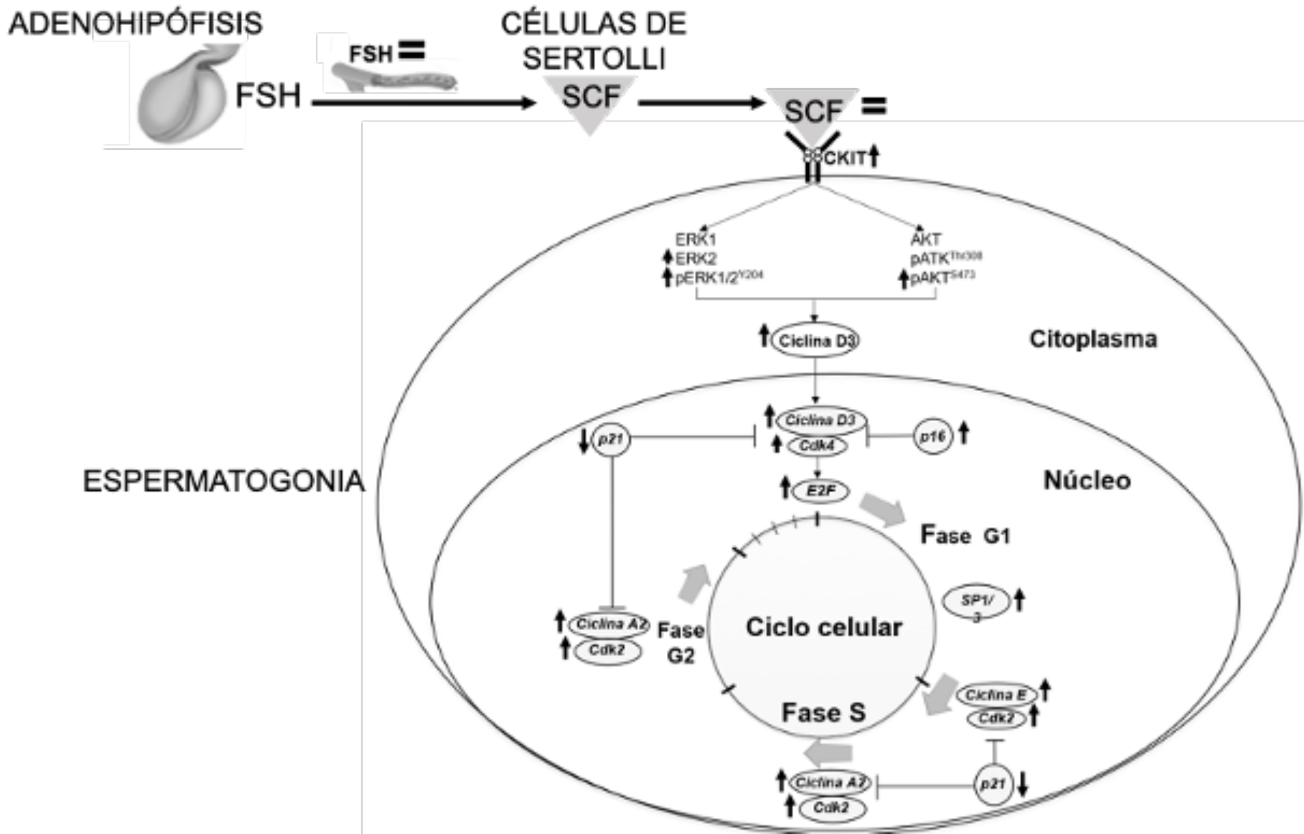

Figura 7. Modelo propuesto de los efectos de la dieta suplementada con biotina en la proliferación de las espermatogonias. La administración de la dieta suplementada con biotina durante 8 semanas incrementa la proliferación de las espermatogonias por un mecanismo en el que participan la expresión del receptor c-kit, las formas activas de las proteínas de señalización ERK/AKT, quienes, mediante la ciclina D3, activan el ciclo celular al nivel de la fase G1⁄S por un aumento del RNAm de la *Ciclina D3, Cdk4, Ciclina E, Cdk2, Ciclina A2*, y el factor *E2F*. En sintonía con su rol inhibidor la expresión del RNAm de p21 disminuye; sin embargo, no acorde con su rol inductor del ciclo celular p16 se observó disminución. Estos efectos están asociados con un aumento en los transcritos de los factores transcripcionales *Sp1* y *Sp3* y el incremento de la expresión proteica del receptor c-kit-receptor. La proliferación celular es independiente de la hormona folículo estimulante FSH y del factor de células troncales SCF.

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Dietary biotin supplementation increases proliferation pathways in mice testes without affecting serum follicle-stimulating hormone levels and stem cell factor expression

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ABSTRACT

Supplements containing pharmacological concentrations of biotin are commercially available. The mechanisms by which biotin at pharmacological concentrations exerts its action have been the subject of multiple investigations, particularly for biotin's medicinal potential and wide use for cosmetic purposes. Several studies have reported that biotin supplementation increases cell proliferation; however, the mechanisms involved in this effect have not yet been characterized. In a previous study, we found that a biotin supplemented diet increased spermatogonia proliferation. The present study was focused on investigating the molecular mechanisms involved in biotin-induced testis cell proliferation. Male BALB/cAnNHsd mice were fed a control or a biotin-supplemented diet (1.76 or 97.7 mg biotin/kg diet) for eight weeks. Compared with the control group, the biotin supplemented mice presented augmented protein abundance of the c-kit-receptor and pERK1/2^{Tyr204} and pAKT^{Ser473}, the active forms of ERK/AKT proliferation signaling pathways. No changes were observed in the testis expression of the stem cell factor and in the serum levels of the follicle-stimulating hormone. Analysis of mRNA abundance found an increase in cyclins *Ccnd3, Ccne1, Ccna2;* Kinases *Cdk4, Cdk2; and E2F;* and *Sp1 & Sp3* transcription factors. Decreased expression of cyclin-dependent kinase inhibitor 1a (*p21*) was observed but not of *Cdkn2a* inhibitor (*p16*). The results of the present study identifies, for the first time, the mechanisms associated with biotin supplementation-induced cell proliferation, which raises concerns about the effects of biotin on male reproductive health because of its capacity to cause hyperplasia, especially because this vitamin is available in large amounts without regulation.

1. Introduction

Besides being a covalently bound coenzyme of carboxylases, it is well-documented that pharmacological concentrations of biotin modify gene expression and have pleiotropic effects (Riveron-Negrete and Fernandez-Mejia, 2017; Zempleni et al., 2009). A variety of biological functions, such as metabolism, reproduction, and development are modified by biotin supplementation (Riveron-Negrete and Fernandez-Mejia, 2017; Zempleni et al., 2009). In the last two decades, efforts have been directed toward identifying the molecular mechanisms by which biotin exerts its effects on biological processes, particularly because of the therapeutic potential of biotin supplementation in the treatment of diabetes, metabolic syndrome, and multiple sclerosis, as also among others, animal husbandry (Mock, 2017; Riveron-Negrete

Abbreviations: AKT, Protein kinase B (PKB); *Ccna2*, Cyclin A2 gene; *Ccne1*, Cyclin E gene; *Ccnd3*, Cyclin D3 gene; *Cdk2*, Cyclin-dependent kinase type 2 gene; *Cdk4*, Cyclin-dependent kinase type 4; c-kit-Receptor, Mast/stem cell growth factor receptor; *E2F*, Transcription factor E2F gene; ERK1, Extracellular signal–regulated kinases Type 1; ERK2, Extracellular signal–regulated kinases Type 2; FSH, Follicle stimulating hormone; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; *p16*, Cyclin-dependent kinase inhibitor type 2A gene; *p21*, Cyclin-dependent kinase inhibitor type1 gene; pAKT^{Ser473}, Phosphorylated AKT at Serine 473 residue; pAKT^{Thr308}, Phosphorylated AKT at Threonine 308 residue; pERK1/2^{Tyr204}, Phosphorylated Extracellular signal–regulated kinases Type 1 & 2; SCF, Stem Cell Factor; SE, Standard error; *Sp1*, Specificity protein 1 gene; *Sp3*, Specificity protein 3 gene; *Tuba1a*, Alpha tubulin gene.

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and Fernandez-Mejia, 2017).

In previous studies, we found that a biotin-supplemented diet increases tissue cellular content and modifies tissue morphology (Pastén Hidalgo et al., 2020; Riverón-Negrete et al., 2016; Lazo de la Vega-Monroy et al., 2013), without changing toxicity markers (Riverón-Negrete et al., 2016), questioning the safety of pharmacological concentrations of the vitamin. In the liver, mice fed a biotin-supplemented diet during eight weeks after weaning showed morphological changes; these modifications were not associated with raises in oxidative stress markers, such as malondialdehyde and glutathione, or liver damage enzymes. In the pancreas, one-week of biotin-supplementation after weaning produced an increase in islet number, area and proportion of beta-cells. These effects were related to an increase in beta-cell augmentation in the Ki67 proliferation marker (Tixi-Verdugo et al., 2017). In the testes, eight weeks of biotin supplementation produced striking effects on the histology of spermatozoa and mouse testis augmenting the number of abnormal seminiferous tubules with non visible lumen and the quantity of intratubular spaces. Furthermore, the supplemented group showed increased number of elongated seminiferous tubules, which presented cellular disorganization, modification in the germinal epithelium and more than three spermatogonia layers, due to increased spermatogonia proliferation (Pastén-Hidalgo et al., 2020).

The possibility that pharmacological concentrations of biotin affect cell proliferation raises concerns about the use of this vitamin since supplements containing biotin concentrations with several orders of magnitude surpassing the human vitamin daily recommendation of 30 μg/day are commercially available in larger amounts without regulation, and mutagenic classical toxicity tests (Hayes et al., 1984; SRI International, 1979) and other studies (Riveron-Negrete and Fernandez-Mejia, 2017) have suggested that biotin administration is harmless. Furthermore, the information on adverse effects from biotin intake have been too scarce and a tolerable upper intake level (UL) has not been derived (Walter, 2001; Australian Government Department of Health and Aging, 2005). Hence, studies documenting the effects and mechanisms addressing the effects of pharmacological concentrations of biotin will provide important information that may help to document the actions of biotin supplementation and to establish the UL values.

The effects of pharmacological concentrations on cell proliferation remain controversial: (Crisp et al., 2004; Griffin et al., 2003; Manthey et al., 2002; Valenciano et al., 2002; Zempleni et al., 2001). In cultured cell lines, increased cell proliferation was observed (Crisp et al., 2004; Griffin et al., 2003; Manthey et al., 2002). In the T lymphocyte cell line, Jurkat, cultured for four weeks, a transient increase of proliferation rates in the second week was observed, but it returned to basal proliferation rates by the third week (Manthey et al., 2002). In Jar-choriocarcinoma cells, an increased rate of thymidine uptake was observed (Crisp et al., 2004). However, certain other studies have found that biotin supplementation decreases (Zempleni et al., 2001) or does not affect cell proliferation (Valenciano et al., 2002).As described above, recent studies in our laboratory showed that feeding mice with a biotin supplemented diet augments the tissue cellular content (Pastén-Hidalgo et al., 2020; Tixi-Verdugo et al., 2017).

The available information regarding the mechanisms by which pharmacological concentrations of biotin modify cell proliferation and cell cycle regulation is scarce. Microarray studies in peripheral blood mononuclear cells isolated from healthy adults after biotin supplementation (8.8 μmol/day biotin for 21 days) revealed that, based on the cellular localization, a significant amount of the gene products modified by biotin supplementation was related to the cell nucleus (Wiedmann et al., 2004). When classified based on the biological process involved, cell cycle- and proliferation-related transcripts were among the most prominent ones (Wiedmann et al., 2004). Interestingly, the foremost modified transcripts were related to signal transduction; however, this study did not identify those transcripts. Other studies have suggested that biotinylation of histones in human cells regulate cell proliferation (Stanley et al., 2001); however, this issue remains controversial (Bailey et al., 2008; Healy et al., 2009), and the current knowledge base indicates that histone biotinylation on cell proliferation is a natural, albeit rare, histone modification (Kuroishi et al., 2011). In a prior study, we found that eight weeks of a biotin-supplemented diet augmented the number of spermatogonia layers by a mechanism related to proliferation but not to apoptosis (Pastén Hidalgo et al., 2020). In the present investigation, we analyzed the effects of *in vivo* biotin supplementation on cell cycle and transduction signaling proteins known to be involved in cell proliferation in testis, a tissue with high proliferation rates.

2. Experimental procedures

2.1. Animal model and experimental design

Animal handling and procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, DC, USA, 1996). All the procedures were approved by the Ethical Committees for Experimentation at the Instituto Nacional de Pediatría (Project 074/2013) and at the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (Project 138).

We choose as an experimental model the one in which we observed morphological changes in the testes (Pastén-Hidalgo et al., 2020); and was used in pioneer studies to mimic the hypotriglyceridemic and hypoglycemic effects (Lazo de la Vega-Monroy et al., 2013; Larrieta et al., 2010) of biotin observed in humans (Revilla-Monsalve et al., 2006; Dokusova and Krivoruchenko, 1972; Hemmati et al., 2013; Albarracin et al., 2005), without changing toxicity markers (Riverón-Negrete et al., 2016), body weight or food consumption (Báez-Saldaña et al., 2009; Lazo de la Vega-Monroy et al., 2013).

24 BALB/cAnN male mice aged three weeks were obtained from the breeding colony of the animal research facility from the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México that purchased the breeder mice from Envigo (Indianapolis, IN, USA) and maintained the colony in the Institute's animal research facility. On the day of weaning, mice were randomly divided into two groups and were fed one of the following diets: biotin-control (TD-97126) or biotin-supplemented diet (TD-02458) containing 1.76 mg and 97.7 mg of free biotin/kg diet, respectively (Envigo, Indianapolis, IN, USA). The mice were housed in groups of four per cage. The animal procedures and the evaluation of the amount of biotin in the diets was described previously (Aguilera-Méndez and Fernández-Mejía, 2012; Lazo de la Vega-Monroy et al., 2013; Pastén-Hidalgo et al., 2020; Báez-Saldaña et al., 2009).

Groups of four mice were housed in polycarbonate cages model N10-PC (Ancare, Bellmore, NY, USA) bedding with 7090 Teklad Sani-Chips (Envigo, Indianapolis, IN, USA) and were kept under conditions of 12 h light/dark cycles with *ad libitum* water and food. The mice and their respective diets were weighed daily. After eight weeks of diet administration, the mice were deprived of food for 12 h and anesthetized with Sevorane® (Sevoflurane; Abbott Laboratories, Mexico City, Mexico). The blood and testes samples were obtained and processed as described in the following section.

2.2. Follicle-stimulating hormone analysis

Blood was collected from posterior cava accordingly with Parasuraman et al., (Parasuraman et al., 2010) and allowed to clot for 30 min at 20° in 1.5 ml sterile Eppendorff (Axigen, Union City, CA, USA) tubes. The clot was removed by centrifugation at 500 *xg* for 10 min at 4 °C and the resulting supernatant was carefully removed using a Pasteur pipette and transferred into sterile 1.5 ml Ependorff tubes. The sera were stored at −20 °C until used. Follicle-stimulating hormone concentrations were determined by ELISA using a commercial kit in accordance with the manufacturer's instructions and protocols (MBS703380 ELISA kit, MyBioSource, San Diego, CA; U.S.A). The kit's detection range was 4 mIU/ml- 140 mIU/ml. The limit of detection 2.5mIU/ml. Intra-assay CV

%: <15%; Inter-assay: CV%:<15%. Serum samples were run by duplicate. All values obtained were in the detection limits of the standard curve.

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated from testis tissue using TRIzol Reagent in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), as described previously (Moreno-Méndez et al., 2019). The RNA quality was analyzed by agarose gel electrophoresis analysis of total RNA, and by spectrophotometry measuring 260/280 ratio (BioTek, Winooski, VT, USA) all samples presented optical density ratios between 1.9 and 2.1. A single strand cDNA was synthesized from 1 μg of total RNA using reverse-transcription reaction with 500 units of M-MVL RT (Invitrogene, Carlsbad, CA, USA) and random hexamers (Invitrogene, Carlsbad, CA, USA). The cDNA was mixed with Sybr Green Master mix (Applied Biosystems, Woolston, Warrington, UK) and a sequence of specific primers; Table 1 provides information on the primers used in this study. The mRNA was determined by real-time quantitative polymerase chain reaction using an ABI Prism 7700 Sequence Detector (Foster City, CA, USA). The quantitative expression of the genes was calculated from the cycle threshold (CT) value of each sample using the relative quantification method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001). All the samples were assayed in triplicate and corrected for tubulin RNA used as internal standard. The mean value for the biotin-supplemented cells was normalized to the expression of control cells, as previously described (Moreno-Méndez et al., 2019).

Table 1
List of q-RT-PCR primers.

Gene symbol	Accession number	Sequence 5′→3′	Product
Tuba1a	NM_011653.2	Fwd: GTG GCA ATG TGT GCT TTC ATA C Rev.: TAA GTG AAA TGG GCA GCT TGG	79
Ccna2	NM_009828.3	Fwd: CCC TGC ATT TGG CTG TGA AC Rev.: ATA GCA GCC GTG CCT ACA AG	91
Ccne1	NM_007633.2	Fwd: TTC GGG TCT GAG TTC CAA GC Rev.: TTC TGG AGC GGA GTG AAA GG	113
Cdk2	NM_016756.4	Fwd: TGT GGT ACC GAG CAC CTG AA Rev.: CGG GTC ACC ATT TCA GCA AA	100
Ccnd3	NM_001081635.1	Fwd: CTC GGC CTG AAA TGC TCT GG Rev.: GCT CCA TCC ACT GCC ATC ATT C	77
Cdk4	NM 001355005.1	Fwd: CGA GCG TAA GGC TGA TGG AT Rev.: TGT CCT CAG GTC CTG GTC TAT	99
Cdkn2a	NM_001040654.1	Fwd: CGA ACT CGA GGA GAG CCA TC Rev.: TAC GTG AAC GTT GCC CAT CA	182
Cdkn1a	NM 001111099.2	Fwd: CAC AGC TCA GTG GAC TGG AA Rev.: ACC CTA GAC CCA CAA TGC AG	111
E2F	NM_001291105.1	Fwd: TTG ACT GAC TGC CTT GCC TGT Rev.: GTG ACA ACA CCC AGC TCA CA	177
Sp1	NM 013672.2	Fwd: ACC AGG TGC AAA CCA ACA GA Rev.: AGA GCT GGG AGT CAG GGT AG	223
Sp3	NM_0001018042.3	Fwd: AAG AAG GTG GTG GGA GAG GT Rev.: GCA GGT GTG CTC TCA GAT GT	111

2.4. Spermatogonia isolation

The dissociation of spermatogonia cells was performed using enzymatic digestion of type-I collagenase and trypsin following the procedure reported by Liu et al., 2011 (Liu et al., 2011). Briefly, one testis of each mouse was minced into small pieces and rinsed twice with Hank's washing solution containing penicillin 200 U/ml and streptomycin 200 mg/ml. The tissue was digested at room temperature with 1 g/L type-I collagenase with moderate agitation for 5 min, let sit for 5 min and centrifuged at 4500 *g* for 5 min. The supernatant was removed and the remaining pellet was treated with 0.25% trypsin diluted in a volume of one fold that of testis digested tissue at 37 °C for 5 min of gentle agitation. The dissociated cells were centrifuged, and filtered a nylon mesh filter. The recovered cells were suspended in DMEM culture media (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). The collected cell suspension was added to the Percoll gradient of 11%, 19%, 27%, 35%, and 43% from the highest concentration to the lowest concentration and centrifuged at 353 *g* for 30 min. Cells located in the Percoll gradient concentration ranging between 27% to and 35%, which correspond to spermatogonia, were collected, washed twice with PBS, and resuspended in PBS adjusting cell density to 1×10^5/ml. Volumes of 200 μl of spermatogonia suspension were lysed in RIPA (Thermo Scientific, St. Peters, MO, USA lysis buffer containing 1% of protease inhibitor (Roche, Mannheim, Germany). The lysed samples were centrifuged at 12,500 *g*, for 15 min at 4 °C, and the supernatant was analyzed by loading a volume of 30 μl containing 2.5×10^4 cells of each sample on preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 6% polyacrylamide.

2.5. Western blot analysis

One testis from each mouse was homogenized with a polytron (Kinematica AG Polytron PT 2100, Luzern, Switzerland) in ice cold lysis buffer containing 50 mM HEPES, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 25 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM dithiothreitol, 0.1% TritonX 100 (Sigma, St. Louis, MO, USA), and protease inhibitor cocktail (Roche, Mannheim, Germany) and centrifuged at 12,500 xg for 30 min at 4 °C.

Total protein was quantified using a Bradford dye-binding protein method (Bio-Rad, Richmond, CA, USA) in accordance with the manufacturer's instructions using BSA as the standard. Next, 30 μg of total protein per sample was boiled in Laemmli sample buffer (Bio-Rad, Richmond, CA; USA) for 5 min and then electrophoresed on preparative sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). 6% polyacrylamida for c-kit, 10% for ERK1/2p^{Tyr204}, ERK1, ERK2, AKT^{Ser473}, AKT^{Thr308}, AKT and GAPDH, and 15% for Cyclin D3 and ERK1. The proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), as described previously (Aguilera-Méndez and Fernández-Mejía, 2012) and identified with specific antibodies (Table 2).

2.6. Statistical analysis

All results were expressed as the mean ± SE. Statistical evaluation was performed using a Student's *t*-test. The GraphPad Prism 6.0 software (Berkeley, CA, USA) was used for the statistical analysis. *P* values of less than 0.05 were considered statistically significant.

Table 2
List of antibodies used in western blotting.

Antibody	Dilution	Trademark	#Catalogue	City, Country
CKIT (E-3) mouse monoclonal	1:1000	Santa Cruz Biotechnology, Inc.	sc-365,504	Santa Cruz, CA, USA
ERK1 (C-16) rabbit polyclonal	1:2500	Santa Cruz Biotechnology, Inc.	sc-93	Santa Cruz, CA, USA
ERK2 (C-14), rabbit polyclonal	1:2500	Santa Cruz Biotechnology, Inc.	sc-154	Santa Cruz, CA, USA
pERK1/2^{Tyr204}(E-4) mouse monoclonal	1:3000	Santa Cruz Biotechnology, Inc.	sc-7383	Santa Cruz, CA, USA
Ki67 mouse monoclonal	1:2000	Santa Cruz Biotechnology, Inc.	sc-23,900	Santa Cruz, CA, USA
Donkey anti goat IgG, HRP-linked Antibody	1:5000	Cell Signaling Technology, Inc.	sc-2020	Santa Cruz, CA, USA
GAPDH (D16H11) rabbit monoclonal	1:2000	Cell Signaling Technology, Inc.	#8884S	*Danvers*, MA, USA
AKT rabbit polyclonal	1:3000	Cell Signaling Technology, Inc.	#9272S	*Danvers*, MA, USA
pAKT^{Ser473}(Ser473) rabbit polyclonal	1:2500	Cell Signaling Technology, Inc.	#9271S	*Danvers*, MA, USA
pAKT^{Thr308} (C31E5E) rabbit polyclonal	1:3000	Cell Signaling Technology, Inc.	#2965S	*Danvers*, MA, USA
Horse Anti-mouse IgG HRP-linked Antibody	1:5000	Cell Signaling Technology, Inc.	#7076	*Danvers*, MA, USA
Goat Anti-rabbit IgG, HRP-linked Antibody	1:5000	Cell Signaling Technology, Inc.	#7074	*Danvers*, MA, USA
Donkey anti-goat IgG, HRP-linked Antibody	1:5000	Santa Cruz Biotechnology, Inc.	sc-2020	*Santa* Cruz, CA, USA

3. Results

3.1. Effect of biotin supplementation on body weight and food consumption

Body weight and food consumption were assessed. Both groups showed a steady weight gain over the eight weeks of diet administration without significant differences between groups (Supplemental Fig. 1, panel A). The amount of food intake/body weight ratio was not significantly different between the groups (Fig. 1, panel B). Neither control nor biotin-supplemented mice showed physical or behavioral changes over the course of the treatment.

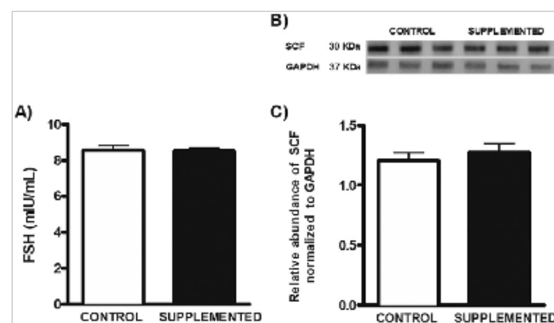

Fig. 1. Effect of biotin supplementation on serum levels of follicle-stimulating hormone and stem cell factor. Panel A. Quantification of serum levels of follicle stimulating hormone concentrations. Bars represent the mean ± SE of 16 mice per group. Panel B. Representative western blot analysis of stem cell factor protein and GAPDH mass. Panel C. Quantification of stem cell factor ligand protein mass normalized to GAPDH. Bars represent the mean + SE of values from 8 mice of each group.

3.2. Effect of biotin supplementation on serum levels of follicle-stimulating hormone and testes stem cell factor

This study intended to evaluate whether biotin's effect on spermatogonia proliferation was related to changes in the levels of follicle-stimulating hormone. As depicted in Fig. 1A, no difference between the groups was found in terms of the serum concentration of this hormone (control: 8.55 ± 0.23; biotin-supplemented: 8.52 ± 0.16 mIU/ml; $P = 0.90$).

The stem cell factor is produced and secreted in the testes under follicle-stimulating hormone's stimulus (Rossi et al., 2000). In accordance with the lack of effect of biotin supplementation on this hormone levels, our data revealed that the protein expression of the stem cell factor in the testes did not differ between the control and the supplemented mice (control: 1.21 ± 0.06; biotin-supplemented: 1.28 ± 0.070; $P = 0.46$) (Fig. 1B).

3.3. Effect of biotin supplementation on the mRNA expression of cell cycle genes

The analysis of the effect of biotin supplementation on the expression of cell cycle proliferation (Fig. 2) revealed that, compared with the control group, eight weeks of biotin supplementation enhanced the transcript abundance of Cyclin D3 (0.68 ± 0.17 fold induction; $P = 0.003$) and Cdk4 (0.55 ± 0.18 fold induction; $P = 0.011$); the RNA expression of *Cyclin E* was increased (0.32 ± 0.11 fold induction ($P = 0.030$) and *Cdk2* was augmented 0.58 ± 0.21 fold induction; $P = 0.016$). Biotin supplementation doubled *Cyclin A2* transcript (0.94 ± 0.037 fold induction; $P = 0.021$) and a fold induction of 1.81 ± 0.80 ($P = 0.041$) was observed in the mRNA expression of *E2F*, a transcription factor of positive action. In accordance with the increased proliferation produced by biotin supplementation, a decrease in the *p21* cyclin-dependent kinase inhibitor was observed, but an increase in *p16*-inhibitor was found (biotin-supplemented: 2.99 + 1.42 fold induction; $P = 0.051$).

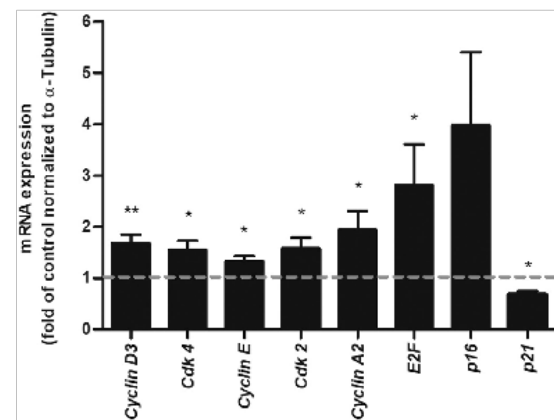

Fig. 2. Effect of biotin supplementation on the mRNA expression of cell cycle genes. Quantification of *Cyclin D3*, *Cdk4*, *Cyclin E*, *Cdk2*, *Cyclin A2*, *E2F*, *p16*, and *p21* mRNA levels normalized to alpha-tubulin; expression levels are presented as fold changes for biotin supplemented cells relative to control values. White bars: control group; black bars: biotin-supplemented group. White bars: control group; black bars: biotin-supplemented group. Bars represent the mean + SE of 10–12 mice per group. *$P \leq 0.05$; **$P \leq 0.0005$.

3.4. *Effect of biotin supplementation on the mRNA expression of the transcription factors: specificity protein1 (Sp1) and specificity protein3 (Sp 3)*

We investigated the mRNA expression of *Sp1* and *Sp3* transcription factors, proteins that are associated with cell proliferation (Safe et al., 2014), whose expressions have been shown to be affected by biotin (Griffin et al., 2003). As shown in Fig. 3, compared with the expression in the control group, *Sp1* and *Sp3* transcripts were increased in the biotin-supplemented group: *Sp1* (0.70 ± 0.19 fold induction; $P = 0.004$) and *Sp3* (1.67 ± 0.28 fold induction; $P = 0.0001$).

3.5. *Effect of biotin supplementation on proteins of the MAPK & PI3K/AKT signaling pathways*

MAPK y PI3K/AKT signaling pathways converge into the activation of Cyclin D3 (Bayascas and Alessi, 2005; Braydich-Stolle et al., 2007; Dolci et al., 2001; Feng et al., 2000; Fu et al., 2018; Rossi et al., 2000; Wei et al., 2018). Therefore, we sought to investigate the involvement of these signals in biotin-induced proliferation of testis cells. As shown in Fig. 4, augmentation in mRNA abundance resulted in increased protein expression of Cyclin D3 (control: 3.14 ± 0.41; biotin-supplemented: 4.62 ± 0.42; $P = 0.030$). Moreover, our data revealed that the active form of ERK and the proliferation-related AKT form, AKT^{Ser473}, were enhanced in the testes of biotin-supplemented mice: $pERK1/2^{Tyr204}$ (control: 1.14 ± 0.19; biotin-supplemented: 2.14 ± 0.23; $P = 0.005$); $pAKT^{Ser473}$ (control: 0.62 ± 0.31; biotin-supplemented: 1.95 ± 0.22; $P = 0.001$). The protein abundance of total ERK2 was increased (control: 1.04 ± 0.025; biotin-supplemented: 1.47 ± 0.16; $P = 0.003$). However, no changes were observed in the protein abundance of ERK1, AKT, and $pAKT^{Thr308}$ forms.

3.6. *Effect of biotin supplementation on c-kit protein abundance*

The canonical proliferation pathways, MAPK and PI3K/AKT in the testes are activated by the paracrine signaling between c-kit-receptor and its ligand, the stem cell factor (Dolci et al., 2001; Feng et al., 2000; Rossi et al., 2000). As shown in Fig. 5A and B, an increase was observed in the testes protein expression of c-kit-receptor (control: 0.86 ± 0.16; biotin-supplemented: 2.03 ± 0.38; $P = 0.012$). Since in previous studies (Pastén-Hidalgo et al., 2020) we found that eight weeks of biotin-supplemented diet increased the number of spermatogonia (Pastén-Hidalgo et al., 2020), we also compared c-kit protein expression levels in protein samples with equal number of a purified population of spermatogonia cells. As shown in Fig. 5C and D, the receptor abundance was increased by 23% (control: 0.86 ± 0.13; biotin supplemented: 1.06 ± 0.05); $P = 0.038$).

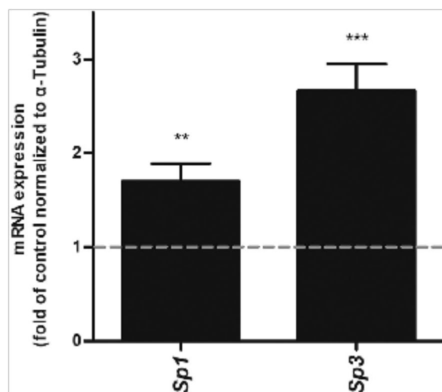

Fig. 3. Effect of biotin supplementation on the mRNA expression of the transcription factors specificity protein 1 (*Sp1*) and specificity protein 3 (*Sp3*). Quantification of *Sp1* and *Sp3* mRNA levels normalized to alpha-tubulin. Expression levels are presented as fold changes for biotin supplemented cells relative to control values. White bars: control group; black bars: biotin-supplemented group. Bars represent the mean ± SE of values from 10 to 12 mice of each group. $^{**}P \leq 0.005$; $^{***}P \leq 0.0005$.

4. Discussion

It is well documented that pharmacological concentrations of biotin have pleiotropic effects, which are related to changes in gene expression at the transcriptional, translational, and post-translational levels (Mock, 2017; Riveron-Negrete and Fernandez-Mejia, 2017; Zempleni et al., 2009). Owing to the therapeutic potential of pharmacological concentrations of biotin (Mock, 2017; Riveron-Negrete and Fernandez-Mejia, 2017), several studies have focused on identifying the molecular mechanisms by which biotin exerts its effects on biological processes. There is scarce information on the effects of biotin on cell proliferation and its mechanisms (Crisp et al., 2004; Griffin et al., 2003; Manthey et al., 2002; Valenciano et al., 2002; Zempleni et al., 2001). We have recently found that eight weeks of a biotin-supplemented diet increased the number of spermatogonia layers in seminiferous tubules and the expression of Ki67 proliferation marker in these cells (Pastén-Hidalgo et al., 2020). In this study, we studied the molecular mechanisms by which biotin supplementation affects cell proliferation in testis cells. We found that the mechanisms involved in increasing the proliferation rate are associated with augmented expression of proteins involved in cell duplication: c-kit-receptor and the activation of MAPK and AKT signaling pathways. We also found that the expressions of several transcripts participating in the cell cycle were modified in response to biotin supplementation. These effects were not mediated by the follicle-stimulating hormone or the stem cell factor.

The follicle-stimulating hormone acts as an inductor of spermatogonia proliferation (Casarini and Crépieux, 2019). Our studies did not find significant differences between the groups in terms of the serum levels of this hormone, indicating that the effects of biotin-supplementation on cell proliferation of testes were not elicited *via* the hypothalamic-pituitary-testicular axis.

The stem cell factor is expressed and secreted in the testes under follicle-stimulating hormone's stimulus (Rossi et al., 2000). In consonance with the lack of effect of biotin supplementation on follicle-stimulating hormone levels, our results found that the protein expression of the stem cell factor did not differ between the control and the supplemented mice. Nevertheless, c-kit – the receptor for the stem cell factor – was found to be significantly augmented in the biotin-supplemented group. In addition, our studies revealed an increase in the active forms of ERK ($pERK1/2^{Tyr204}$) and AKT^{Ser473}, the effector proteins of the signaling pathways associated with c-kit-receptor-mediated cell proliferation, which converge in mediating nuclear translocation of Cyclin D3 and cause the consequent activation of the cell cycle machinery at the G1/S boundary during cell proliferation (Dolci et al., 2001; Feng et al., 2000; Hasegawa et al., 2013).

In the microarray analysis of peripheral blood mononuclear cells isolated from healthy adults after biotin supplementation (Wiedmann et al., 2004), one of the most important changes seen was in the cell cycle and cell proliferation mRNA transcripts; however, the identities of these mRNAs were not reported. In the present study, the examination of the expression of diverse factors involved in the cell cycle machinery revealed that biotin-supplementation increased the mRNA expression of activators of the cell cycle: *Cyclin D3*, *Cdk4*, *Cyclin E*, *Cdk2*, *Cyclin A2*, and the transcription factor *E2F*. In accordance with its role as a cyclin dependent-kinase inhibitor, the mRNA expression of *p21* was found to have decreased. In contrast, an intense augmentation was observed in the mRNA expression of inhibitor *p16*, a protein that prevents progression from G1 phase to S phase. p16 can be activated by reactive oxygen

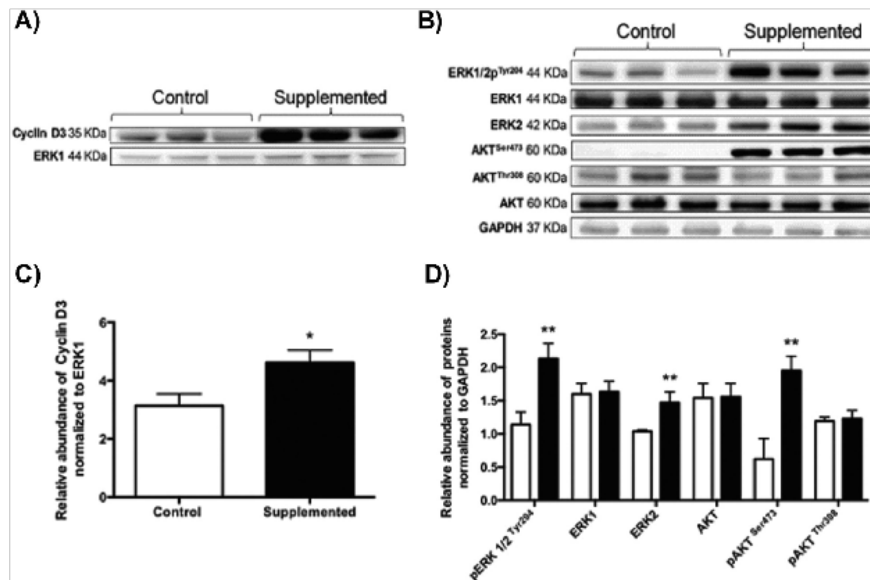

Fig. 4. Effect of biotin supplementation on proteins of the MAPK and PI3K/AKT signaling pathways. Panel A. Representative western blot analysis of Cyclin D3 and ERK1 protein mass; Panel B. Representative western blot analysis of pERK1/2^{Tyr204}, total ERK1, ERK2 and AKT, pAKT^{Ser473}, AKT^{Thr308}, and GADPDH protein mass; Panel C. Quantification of Cyclin D3 normalized to ERK1. White bars: control group; black bars: biotin-supplemented group. Bars represent the means ± SE of values from six mice of each group. *$P \leq 0.05$. Panel D: Quantification of pERK1/2^{Tyr204}, total ERK1, ERK2 and AKT, pAKT^{Ser473}, AKT ^{Thr308} normalized to GADPDH levels. Bars represent the means ± SE of values from eight mice of each group. *$P \leq 0.05$; **$P \leq 0.005$.

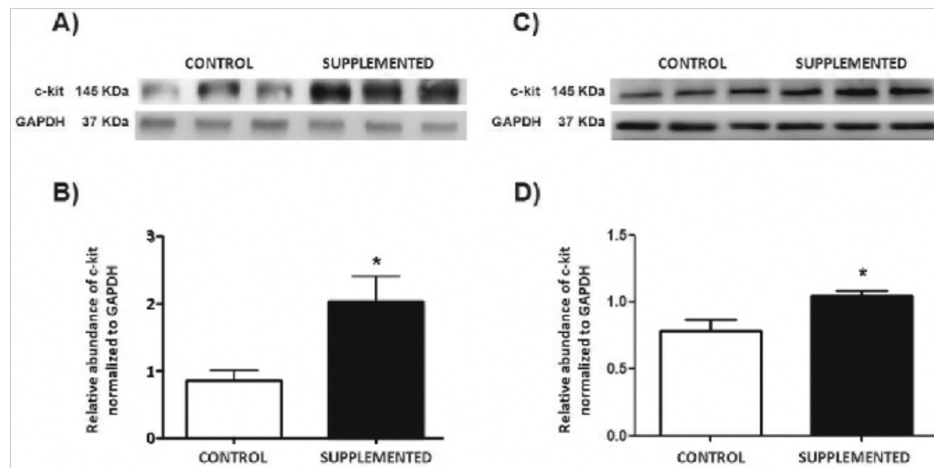

Fig. 5. Effect of biotin supplementation on c kit receptor protein expression. Panel A. Representative western blot analysis of testes expression of c kit and GAPDH protein mass; Panel B. Quantification of testes c-kit receptor normalized to GADPDH. Bars represent the means ± SE of values from 8 mice of each group. *$P < 0.05$. Panel C. Representative western blot analysis of c-kit receptor and GADPDH protein mass in purified population of spermatogonia cells (2.5×10^4 cells) extracts. Panel D. Quantification of c-kit receptor normalized to GADPDH in purified population of spermatogonia protein extracts. White bars: control group; black bars: biotin supplemented group. Bars represent the means ± SE of values from 5 mice of each group. *$P < 0.05$.

species, DNA damage, or senescence, leading to the buildup of this inhibitor in tissues (Kaneto et al., 1999; Qiu et al., 1996; Xin-Chang et al., 2002). Several reports have found that biotin supplementation did not produce oxidant stress in the liver (Aldahmash et al., 2016; Riverón-Negrete et al., 2016; Riveron-Negrete and Fernandez-Mejia, 2017; Sahin et al., 2013), kidney (Aldahmash et al., 2015), and serum (Sahin et al., 2013); however, since the testes are very sensitive to toxicants and drugs (Bonde, 2010; Meistrich, 1986), and have a high capacity for biotin accumulation (Sawamura et al., 2015), we cannot rule out the possibility that biotin supplementation in the testes might produce oxidant stress. Nevertheless, further studies are required to determine the causes of p16 expression increase.

The testes are composed by different cell types, such as spermatogonia, peritubular myoid cells, Sertoli cells, and Leydig cells. c-kit-receptor is predominantly expressed in spermatogonia and is used as a marker of spermatogonia proliferation. In previous studies (Pastén-Hidalgo et al., 2020) we showed that biotin-supplemented diet led to augments in spermatogonia number and proliferation. The present work revealed that in the biotin-supplemented group, the expression of c-kit was increased in protein extracts of testes, as well as in extracts containing equal number of purified population of spermatogonia. However, the increase was higher in the testes preparation, given the increased number of spermatogonia observed in our previous studies. Our present results indicate that increases in C-KIT expression participate in the molecular mechanism of biotin-induced spermatogonia proliferation."

Since the analysis of MAPK and AKT was assessed in the complete testis organ whose different cell-type express these signaling pathways proteins (Lai et al., 2014; Ni et al., 2019; Thuillier et al., 2009; Yamashita et al., 2011), we cannot rule out the possibility that the effects of biotin-supplementation on the expression of signaling proteins in other tissue cell types will also account for the increase observed in the present study. On the other hand, although other testis cell types might participate in the changes observed in the expression of cyclins, they have limited capability, compared to spermatogonia, to proliferate at the age of the mice used for the analysis (Benton et al., 1995; Orth et al., 1988). Moreover, in our prior immunohistological investigations (Pastén-Hidalgo et al., 2020) we showed that biotin-induced proliferation was restricted to spermatogonia, indicating the low likelihood of other testis cells contributing to cyclin expression.

We found that the stem cell factor—the ligand of testes germ cell proliferation—was not modified by the biotin-supplemented diet, suggesting that biotin directly affects the expression of genes of the general proliferation machinery. In support of this view, several studies have found that biotin produces a direct effect on proliferation in cell cultures (Crisp et al., 2004; Griffin et al., 2003; Manthey et al., 2002) and in different cell types (Pastén-Hidalgo et al., 2020; Tixi-Verdugo et al., 2017). Further, according to Griffin et al., (Griffin et al., 2003) the molecular mechanisms involved in biotin-dependent cell proliferation in Jurkat cells are related to the involvement of Sp1 and Sp3 transcription factors, proteins associated with cell proliferation (Safe et al., 2014), which are expressed in the spermatogonia (Ma et al., 2008; Thomas et al., 2005). Sp1 also acts as a transcription factor of mouse c-kit-receptor (Yasuda et al., 1993). Nevertheless, other transcription factors might participate in the spermatogenesis proliferative phase as well. Current studies in our laboratory are exploring this issue in isolated spermatogonia.

Few studies have addressed the effect of pharmacological concentrations of biotin on testes (Sawamura et al., 2015; Sawamura et al., 2007). *In vivo*, studies by Sawamura found that 5000 and 10,000 mg biotin/kg diet intake over six weeks after weaning decreased the number of spermatogonia, and produced apoptosis like cell death in the spermatogenic epithelium of the seminiferous tubules (Sawamura et al., 2015, Sawamura et al., 2007); however, this quantity of biotin was found to decrease body weight and food ingestion and showed to be toxic, resulting in a 50% mortality rate (Sawamura et al., 2015, Sawamura et al., 2007). Thus, it is very likely that the number of spermatogonia decreases at this extremely high concentration of biotin as a result of the vitamin's toxic effects at the systemic level. In contrast, in our experimental model, a diet containing 97.7 mg biotin/kg increased the number of proliferating spermatogonia (Pastén-Hidalgo et al., 2020), but did not affect the body weight and food consumption, as shown in the present diet for eight weeks study (Supplementary Fig. S1) and in previous studies (Báez-Saldaña et al., 2009; Lazo de la Vega-Monroy et al., 2013).

It is important to note that, mutagenic classical toxicity tests (Hayes et al., 1984; SRI-International, 1979) and studies documented biotin cosmetic uses have indicated that biotin administration is harmless (Fiume and Cosmetic Ingredient Review Expert Panel, 2001). Mice fed a biotin-supplemented diet (97.7 mg biotin/kg diet) during eight weeks after weaning did not affect oxidative stress markers, such as malondialdehyde and glutathione in the liver, or serum hepatic damage enzymes. Reports on the effect of biotin supplementation on biochemical toxicity parameters in rats found that markers, such as alanine aminotransferase, aspartate aminotransferase, creatinine and blood urea nitrogen, were not affected by a 100–10,000 mg biotin /kg diet (Sawamura et al., 2015). However, in other studies, the same authors reported that a diet containing 10,0000 mg biotin/kg was toxic (Sawamura et al., 2007). Diarrhea was observed in rats fed a 5000 mg biotin/kg diet (Sawamura et al., 2015). Studies on the LD_{50} in rodents reported intravenous LD_{50} of 1000 mg/kg body weight (Bonjour, 1991) in mice. In rats, biotin's oral and i.p. LD_{50} acute toxicity values are 354 and 29 mg/kg body weight, respectively (Bonjour, 1991). In humans, oral doses up to 100 mg biotin/d to biotinidase- and holocarboxylase synthetase-deficient patients did not cause adverse effects (Baumgartner and Suormala, 1997). Clinical investigations in diabetic patients reported that 9 mg of biotin daily during 48 months did not aggravate diabetes or produced undesirable side effects (Maebashi et al., 1993). Biotin doses 1.5–3 mg for 1–2 years were well tolerated in patients suffering from diabetic peripheral neuropathy (Koutsikos et al., 1990). In patients undergoing hemodialysis, no adverse effects were found with intravenous administration of 50 mg biotin three times per week for two months (Koutsikos et al., 1996). However, none of the studies described above analyzed the effects of biotin on male reproductive features. Though, in a recent report on the side effects of biotin, it was reported that two people from 22,547 individuals (*i.e.*, 0.01%) reported to have testicular pain (https://www.ehealthme.com/ds/biotin/testicular-pain/). Toxicology tests, such as whole life-cycle and multi-generation tests that would detect changes in reproductive outcomes and reproductive organ pathology have not yet been evaluated.

Our present studies showing the effects of biotin supplementation on cell cycle molecules, in the same experimental model in which we observed favorable effects on glucose and lipid homeostasis (Aguilera-Méndez and Fernández-Mejía, 2012; Larrieta et al., 2010; Lazo de la Vega-Monroy et al., 2013), and no changes on toxicity markers (Riverón-Negrete et al., 2016) raises concern regarding the effects of pharmacological concentrations of biotin on male health, since several studies have found that the majority of male germ cell tumors overexpress cell cycle activators, such as Cyclin A2, and Cdk2 (Liao et al., 2004), whose transcripts were found to be increased by biotin supplementation in the present study. Furthermore, these results, along with our recent finding (Pastén-Hidalgo et al., 2020) that biotin-supplementation impairs testis morphology and has negative effects on spermatozoa structure and motility, strongly caution against the use of supplements with high concentrations of biotin, which are commercially available without regulation and whose cosmetic uses have escalated considerably in recent years (John and Lipner, 2019), despite the lack of sufficient clinical evidence supporting its efficacy (Soleymani et al., 2017). It is important to note that serum biotin levels attained by supplements with high levels of biotin (5–10 mg) (Pastén-Hidalgo et al., 2020; Riveron Negrete and Fernandez Mejia, 2017; Manthey et al., 2002) could reach the serum vitamin concentration in the same order of magnitude observed in our present experimental model (Lazo de la Vega-Monroy et al., 2013; Mock and Mock, 1997).

In summary, biotin supplementation increased the c-kit-receptor and ERK/AKT signaling protein's expression as well as the mRNA expression of *Sp1* and *Sp3* and cell cycle transcription factors; this effect was independent of the effect of follicle-stimulating hormone and the stem cell factor (Fig. 6). The present work identifies, for the first time, the mechanisms involved in producing the effects of biotin supplementation on cell proliferation. Moreover, the results raise caution regarding the potential risks of biotin induced testis cell proliferation.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2021.115774.

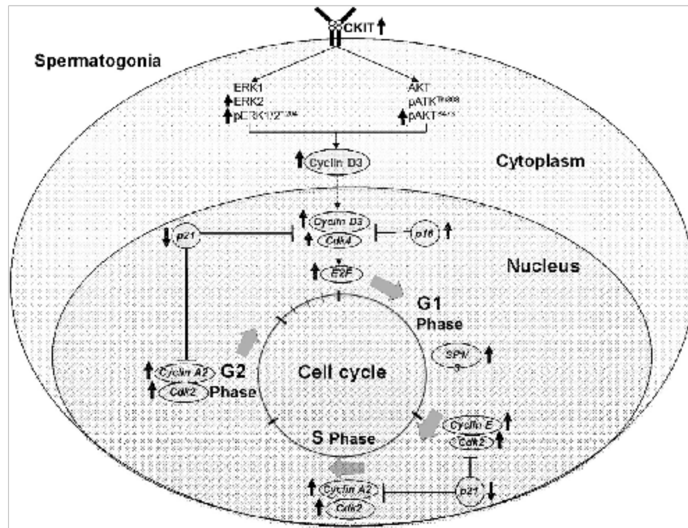

Fig. 6. Proposed model of the mechanisms involved in the effects of biotin-supplementation on testes cell proliferation. Biotin supplementation increased spermatogonia proliferation through a mechanism involving increased protein expression of c-kit receptor, the active forms of ERK/AKT signaling proteins, and Cyclin D3, with the consequent activation of the cell cycle machinery at the G1 / S boundary during cell proliferation; mRNA expression of activators of the cell cycle, *Cyclin D3, Cdk4, Cyclin E, Cdk2, Cyclin A2*, and the transcription factor *E2F*, were increased. In accordance with its role as a cyclin dependent-kinase inhibitor, the mRNA expression of p21 was decreased, and notwithstanding with the induction of cell cycle proliferating cyclins, p16 was decreased; these effects were associated with augmented mRNA expression of *Sp1* and *Sp3* transcription factors and increased c-kit-receptor expression, proteins associated with spermatogonia proliferation. Cell proliferation was independent of serum follicle-stimulating hormone levels and the stem cell factor protein expression.

CRediT authorship contribution statement

Tonatiuh Salazar-Anzures: Conceptualization, Investigation, Methodology, Formal analysis, Writing – original draft. **Karina Pastén-Hidalgo:** Conceptualization, Investigation, Methodology, Formal analysis. **Gloria Sicilia-Argumedo:** Resources, Methodology. **Leticia Riverón-Negrete:** Methodology, Investigation, Formal analysis. **Alain de Jesús Hernández-Vázquez:** Formal analysis, Data curation. **Cristina Fernanadez-Mejia:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

We acknowledge that the authors Tonatiuh Salazar-Anzures, Karina Pastén-Hidalgo, Gloria Sicilia-Argumedo, Leticia Riverón Negrete, Alain Hernández-Vázquez and Cristina Fernandez-Mejia have no financial interest or other contractual agreements that might cause conflicts of interest or be perceived as causing conflicts of interest in any company or organization sponsoring the research.

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Dietary Biotin Supplementation Impairs Testis Morphology and Sperm Quality

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ABSTRACT Supplements containing pharmacological concentrations of biotin are commercially available over the counter. Classical toxicity studies have considered biotin administration as harmless; however, recent investigations have shown that biotin supplementation modifies tissue morphology without changes in toxicity markers, raising concerns about the consequences of morphological changes on tissues' functions and the safety of pharmacological concentrations of the vitamin. Testes are very sensitive to toxicants, and testicular histology is a reliable method to study its function. In this work, we investigated the effects of dietary biotin supplementation on testis morphology and spermatogenesis function using an experimental model, in which we have not observed unfavorable effects on other tissue functions or toxicity markers. Male BALB/cAnNHsd mice were fed a control or a biotin-supplemented diet (1.76 or 97.7 mg biotin/kg diet) for 8 weeks. Compared to the control group, the biotin-supplemented mice presented remarkable testis morphology changes, including increased spermatogonia layers; the cellular mechanism involved is related to increased proliferation. Sperm count and serum testosterone levels were not affected, but spermatozoa motility and morphology were significantly impaired in the biotin-supplemented mice. These results caution against the use of supplements with high concentrations of biotin and indicate that biotin's pharmacological effects on morphology need to be considered in toxicological studies.

KEYWORDS: • ***proliferation*** • ***spermatozoa*** • ***toxicity*** • ***vitamin B***

INTRODUCTION

Biotin is a B-complex vitamin whose physiological role is to act as a covalently bound coenzyme of carboxylases. At present, it is well accepted that pharmacological concentrations of biotin modify biological processes.[1,2] Several reports have documented that pharmacological concentrations of biotin modify the expression of genes involved in glucose and lipid homeostasis, have hypolipemic effects, and decrease hyperglycemia.[1,2] The effects of biotin on these functions indicate its potential to be used in strategies to managing diabetes and dyslipidemia. Indeed, biotin products with pharmacological concentrations of the vitamin are commercially available for different proposes—for example, nail and hair health, pregnancy and breastfeeding, and to reduce blood glucose in people with diabetes. Despite its use in elevated amounts, the highest average level of daily intake that is likely to pose no risk of adverse health to most individuals (tolerable upper intake level of a nutrient [UL]) have not been established.[3,4]

Recent studies showing that biotin supplementation modifies tissue architecture without changes in toxicity markers have raised concern about the consequences of morphological changes on tissues' functions and the safety of pharmacological concentrations of biotin.[5] We found that mice fed a biotin-supplemented diet during 8 weeks showed an increased proportion of nucleomegaly and binucleated hepatocytes, altered portal triads with increased dilated sinusoids (27%), increased vascularity (37%), and increased number of bile conducts (19%); these modifications were not associated with increases in classical liver damage indicators and oxidative stress markers, such as malondialdehyde, glutathione, urea, creatinine, and serum liver enzymes.[5] These results indicate that the studies to

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determine the safeness of pharmacological concentrations of biotin need to take into consideration its effects on tissue morphology.

Other studies support the action of biotin pharmacological concentrations on tissue structure. In animal models of hyperglycemia, biotin supplementation ameliorated the pathological changes in the cellular architecture of the pancreas,[6] kidney,[6] and liver[6,7] in the diabetic animals. In the pancreas of normal mice, we found that 8 weeks of a biotin-supplemented diet increased islet size and changed its typical architecture of alpha-cells at the periphery and beta-cells at the core.[8] In female mice, our studies found that a biotin-supplemented diet decreased both Graafian and ovarian primary follicle numbers.[9] Atrophy of the corpus luteum and ovary stroma were found in rats injected with 50 mg of biotin/kg body weight.[10]

The effects of biotin supplementation on the male reproductive system have been poorly investigated. In male rats, Sawamura *et al.*[11,12] investigated the effects of biotin-supplemented diets on testes, and did not detect changes in testis weight in rats fed a diet containing 100 mg/kg diet or 1000 mg of biotin/kg diet over 6 weeks after weaning.[11] In other studies,[12] the same investigators found that rats fed 400, 800, 1000, and 2000 mg biotin/kg diets for 28 days did not change testis weight, but with 5000 and 8000 mg biotin/kg diets the tissue weight was decreased; unfortunately, they did not analyze testis morphology in these studies.

In rats fed a diet containing 10,000 mg biotin/kg over 6 weeks, the authors found that the weight of the testes decreased by about 75%. Their analysis of testes morphology found that the development of seminiferous tubules was inhibited, and few spermatogonia were observed. Also, the number of mature sperm was markedly lower, and sperm with morphologically abnormal heads, mostly round heads, was increased. However, 10,000 mg biotin/kg diet intake was found to be toxic and caused 50% mortality,[11,12] so it is very likely that the testes changes at this extremely high concentration of biotin resulted from the vitamin's toxic effects at systemic level.

The testes are very sensitive to toxicants, drugs, or radiation,[13,14] and testicular histology is a sensitive and reliable method for studying sperm production.[15] Because the effects of pharmacological concentrations of biotin on tissue morphology raise concern about the consequences for tissue function, in this work, we have investigated the effects of dietary biotin supplementation on testis morphology and function using an experimental model, in which no unfavorable effects on tissue functions or toxicity markers were found.[5,8,9] We hypothesize that this strategy will help to expose possible deleterious effects associated with biotin supplementation and might help to establish biotin's UL.

MATERIALS AND METHODS

Animal model and experimental design

Animal procedures were done according with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, DC, USA, 1996) and approved by the Ethics Committee for Experimentation of the Instituto de InvestigacionesBiomédicas, Universidad NacionalAutónoma de México.

At weaning, 3-week-old male Balb/cAnN mice were fed one of the following diets: biotin-control (TD-97126) or biotin-supplemented diet (TD-02458) containing 1.76 mg and 97.7 mg of free biotin/kg diet, respectively (Harlan Teklad, Madison WI, USA), as described previously.[16] Mice were kept in 12 h light/dark cycles at 22°C ± 2°C and provided water and food *ad libitum*. After 8 weeks of diet administration, the mice were deprived of food for 12 h and anesthetized with Sevoflurane (Abbott Laboratories, Mexico City, Mexico). The blood, testes, and cauda epidydimal sperm were obtained. Finally, mice were killed by cervical dislocation.

Testosterone measurements

After 8 weeks of diet administration, blood samples were obtained from anesthetized mice between 9 and 10 AM and centrifuged at 10,000 *g* and 4°C for 10 min. Sera were stored at −20°C until used. Testosterone concentrations were determined by ELISA using a commercial kit (DRG Testosterone ELISA, GmbH, Germany) according to the manufacturer's instructions.

Morphological and morphometric analysis

One testis from each mouse was fixed in formalin. Consecutive 5 μm-thick sections were cut and stained with hematoxylin and eosin. Slides were examined for histological changes under a light microscope (Olympus IX-71 inverted microscope; Olympus Corporation, Tokyo, Japan). Morphology was assessed according to Bacha and Bacha,[17] and included histopathology markers commonly used to quantify testicular injury, which include evaluation of seminiferous tubule diameters, and sloughing.[18]

We evaluated a total of about 100 tubules randomly selected from three different sections from eight mice. Tubular spaces were considered as unstained spaces inside the tubule. Alteration of cells which surround the seminiferous tubules in the testis was considered as changes in the epithelium basal lamina. Seminiferous tubule circularity was determined in cross-sections of testes using NIH ImageJ software by the formula 4π (area/perimeter^2), a value of 1 indicates the circular shape, and as the value decreases, it denotes elongatation.[19] Tubule elongation degree was determined by measuring the major axis, which goes from one side of the tubule, through the center, to the other side, at the widest part of the tubule. The minor axis is the shortest diameter.[20] Testes structures appearing with a frequency minor to 5% were classified as sporadic anomalies.

The seminiferous degree of maturity was determined by the Jonhsen index,[21] assigning a score to each tubule according to the main cell-type present, ranging from 1 (no cells) to 10 (complete spermatogenesis with more than 10 late spermatids). The number of interstitial Leydig cells

was determined as reported.[22] Sertoli cells were identified by the presence of two nucleoli. The analyses were performed by two persons who were blinded to the group identity. All images were analyzed using ImageJ 1.50 software (National Institute of Mental Health, Bethesda, MD, USA).

Immunofluorescence studies

Proliferation and apoptosis were analyzed by immunofluorescence as described previously.[23] Cell proliferation was assessed with anti-rabbit Ki67 (Abcam #15580, Cambridge, MA, USA). After 1.5 h at 37°C of incubation, slides were washed and incubated for 2 h at room temperature with AlexaFluor-594-conjugated secondary antibody (Abcam #150080). Nuclei were stained with DAPI (Sigma #D9542, St. Louis, MO, USA). As a negative control, the primary antibody was substituted with phosphate-buffered saline (PBS). Seminiferous tubule cell proliferation was measured by quantifying the number of Ki67-positive nuclei in relationship to the number of total nuclei. A total of 127 tubules were counted from each group using ImageJ 1.50 software.

Apoptosis was analyzed using the TUNEL Assay Kit (In situ Cell Dead Detection Kit, fluorescence; Roche Diagnostics #11684795910, Manheim, Germany). Nuclei were counterstained with DAPI, and apoptosis was analyzed by quantifying the number of TUNEL-positive nuclei in relationship to the number of total nuclei. A total of 155 tubules from both groups were counted using ImageJ 1.50 software.

Western blot analysis

The tissue was homogenized and total protein quantified as described previously.[24] Next, 35 μg of protein were electrophoresed on preparative SDS polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane and probed with antibodies against Ki67, caspase-3 (Cell Signaling Technology #96615; Danvers, MA, USA), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH; Cell Signaling Technology #88845). Anti-IgG-HRP conjugates were used as secondary antibodies (Cell Signaling Technology #70765 and 7074). Protein bands were detected in a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA) and quantified by densitometry using ImageJ 1.40 (Research Services Branch, National Institute of Mental Health).

Spermatozoa isolation

Both epididymides were obtained, placed on a prewarmed culture dish, maintained at 37°C. Then, fat and connective tissues were removed.[25] Caudal regions from the epididymis were isolated and minced. Sperm cells were allowed to disperse out of the cauda. Afterward, sperm in suspension were washed in PBS by centrifugation at 600 *g* for 5 min. Finally, sperm cells were counted using a hematocytometer.

Sperm morphology and motility

Spermatozoa were fixed in formaldehyde and counted as previously described.[26] To evaluate flagellar alterations, 1000 spermatozoa from each mouse ($n=8$) strain were classified and counted. Sperm morphology was examined by phase-contrast microscopy (Olympus IX-71 inverted microscope; Olympus Corporation) and classified as follows: (1) with normal flagella, not curved or twisted or (2) with any number of all curved flagella in the middle piece.[26] Sperm motility was analyzed using a light microscope and counting motile and nonmotile spermatozoa and was expressed as motility percent.[27]

Statistical analysis

All data are presented as the mean ± SEM; n denotes the number of evaluated subjects. Statistical analysis was performed using GraphPad (La Jolla, CA, USA). The data were analyzed by Student's t-test. $P \leq .05$ was considered statistically significant.

RESULTS

Effect of biotin supplementation on body and testis weight and serum testosterone concentration

We determined the effect of 8 weeks of biotin supplementation on body and testis weight and hormonal concentration. As shown in Table 1, no differences were observed regarding the body and testis weight, the testis weight to body weight ratio, or the serum testosterone levels between the groups.

Effects of biotin supplementation on testis morphology

No differences between the control and the biotin-supplemented group were observed in the external appearance of the testes (Fig. 1A). Light microscopy of the testes revealed a number of differences between the biotin-supplemented group and the control group (Fig. 1B–D). As seen in Figure 1C, the testis of supplemented mice presented an increased percent of nonvisible seminiferous tubule lumen (control: 22.5% ± 0.94%; biotin-supplemented: 29.4% ± 2.90%; $P<.05$) and in the number of seminiferous tubules with intratubular spaces (control 7.90% ± 2.70%; biotin-supplemented 24.0% ± 3.80%; $P<.005$). Sloughing of round germs in the lumen was observed. The testis interstitial space showed an increase in Leydig cells (control: 16.0% ± 0.42%; biotin-supplemented:

Table 1. Effects of Biotin on Body and Testis Weight, Testis Weight/Body Weight Ratio, and Serum Testosterone Concentration

	Control	*Supplemented*
Body weight (g)	23.3 ± 0.57	22.2 ± 0.61
Testis weight (mg)	183 ± 6.45	187 ± 3.12
Testis weight:body weight (ratio)	0.783 ± 0.003	0.845 ± 0.002
Testosterone levels (ng/mL)	2.42 ± 0.33	1.75 ± 0.25

Values are means ± SEM $n=8$ mice for group.

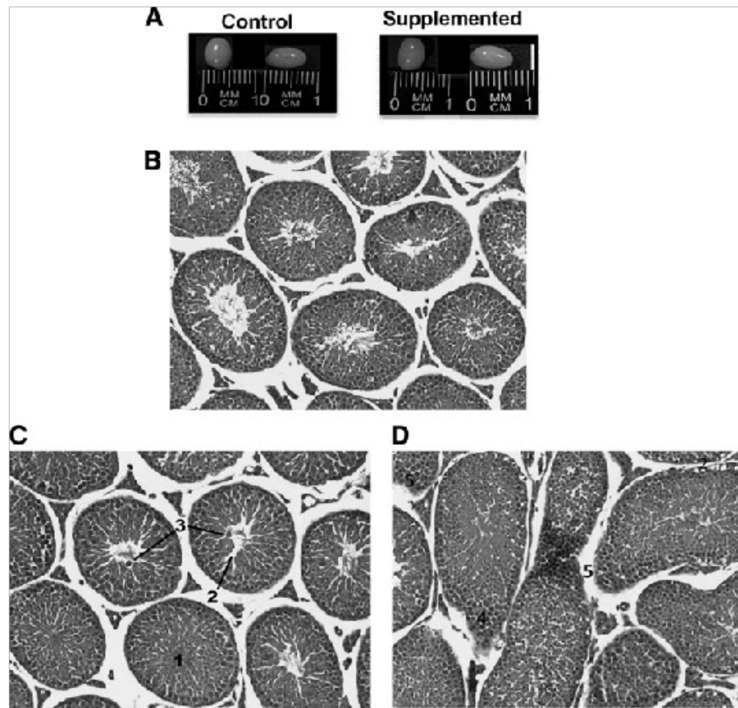

FIG. 1. Effect of biotin supplementation on testis size and morphology. **(A)** Representative images of testes size. Representative images showing paraffin sections of testes stained by hematoxylin/eosin; **(B)** Control group. **(C)** Supplemented group images showing testis anomalies: nonvisible lumen (1); intratubular space (2) sloughing of round germs in the lumen was observed (3). **(D)** Biotin-supplemented group image showing elongated seminiferous tubules with cellular disorganization and more than three spermatogonia layers (4); alteration in the germinal epithelium (5). Scale bar: 50 μm.

19.7% ± 0.67%: P < .0001). Furthermore, the supplemented group (Fig. 1D) presented loss of circularity, increased elongated seminiferous tubules (control: 13.8% ± 2.37%; biotin-supplemented: 49.3% ± 8.14%; P < .0001) with cellular disorganization, more than three spermatogonia layers, and alteration in the germinal epithelium (control: 0.38% ± 0.18% biotin supplemented: 3.50% ± 0.32%; P < .0001). We also found a decrease in the degree of maturity determined by the Jonhsen Index (control: 9.03 ± 0.10; biotin-supplemented: 8.42 ± 0.09; P < .0001). No difference between the groups was observed in the number of Sertoli cells (control: 12.4 ± 0.18; biotin-supplemented: 12.5 ± 0.18).

Testes structures appearing with a frequency minor to 5% in the biotin-supplemented mice included sloughing (2.0% ± 0.21%) (Fig. 2A, B), incomplete spermatogenesis (1.97% ± 0.33%) (Fig. 2C), intratubular spaces in the elongated tubule (1.42% ± 0.33%) (Fig. 2C), and Leydig cell hyperplasia (1.5% ± 0.32%) (Fig. 2 D). None of these structures was found in the control group.

Effect of biotin supplementation on sectional seminiferous tubule circularity

We quantified the loss of seminiferous tubule circularity. Compared to the control group, the biotin-supplemented mice presented decreased circularity (Fig. 3A) (control: 0.93 ± 0.006; biotin-supplemented: 0.78 ± 0.017; P < .0001). Also, we assessed the degree of tubule elongation by measuring the long and short diameter of the seminiferous tubules (Fig. 3B). The long diameter of the seminiferous tubules was higher in the biotin-supplemented mice than in the control group (control: 231 ± 4.29 μm; biotin-supplemented: 353 ± 16.7 μm; P < .0001). No significant statistical difference

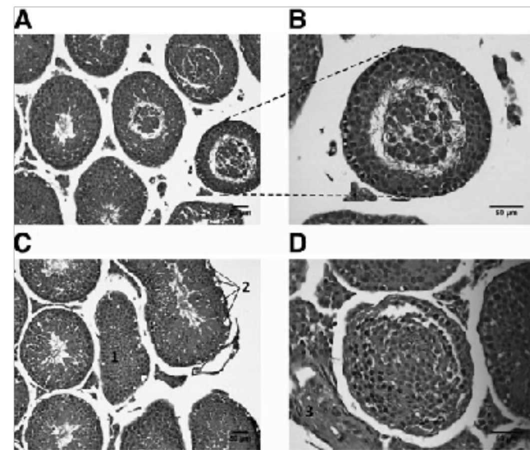

FIG. 2. Effect of biotin supplementation on testis producing sporadic anomalies. **(A)** Disorganized sloughing germ cells. **(B)** Disorganized sloughing germ cells. **(C)** Incomplete spermatogenesis (1); intratubular spaces (2). **(D)** Leydig cells hyperplasia (3). Scale bar: 50 μm.

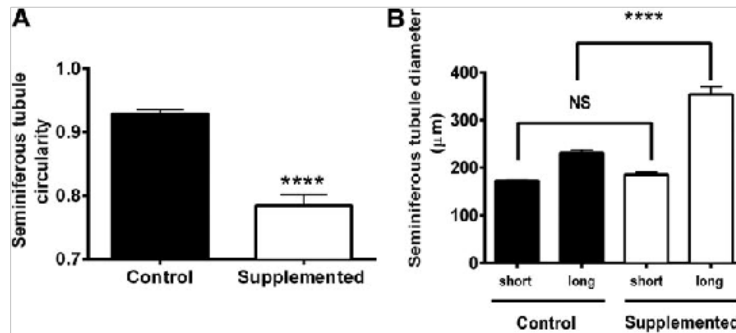

FIG. 3. Effect of biotin supplementation on seminiferous tubule circularity and diameters. **(A)** Seminiferous tubule circularity. *Black bar*: control group; *white bar*: biotin-supplemented group. Values represent the mean ± SEM of eight mice from each group. $^{****}P < .0001$. **(B)** Seminiferous tubule diameters. *Black bars*: control group; *white bars*: biotin-supplemented group. Values represent the mean ± SEM of eight mice from each group. $^{****}P < .0001$; NS, no statistical difference.

between the groups was observed regarding the short diameter (control: 173 ± 1.41 µm; biotin-supplemented: 185 ± 4.29 µm). These data indicate that the shape changes produced by biotin supplementation were due to elongation rather than to a decrease in the tubule diameter.

Effect of biotin supplementation on cell proliferation of testes

We further explored the mechanisms involved in the effects of biotin supplementation on testes. The effect of biotin on cell proliferation was determined by measuring the nuclear expression of Ki67. As shown in Figure 4A, immunofluorescence studies showed that the Ki67 label was increased in the spermatogonia (Fig. 4A). Quantification of spermatogonia immunofluorescent positive nuclei showed an increase in the biotin-supplemented mice (control: 20.3% ± 0.95%; biotin-supplemented: 27.6% ± 1.05%; $P < .0001$) (Fig. 4B). The Ki67 label was also present in the nuclei of Leydig cells (Control: 0.50% ± 0.19%; biotin-supplemented: 0.44% ± 0.18%; $P > .05$), as well as in the cytoplasm. We also analyzed Ki67 protein abundance by western blot (Fig. 4C). Accordingly with the augment observed in the immunofluorescence studies, biotin supplementation increased Ki67 protein expression (Fig. 4D) (control: 0.19 ± 0.016; biotin-supplemented: 0.94 ± 0.047; $P < .001$).

Effects of biotin supplementation on testis apoptosis

Immunofluorescence studies revealed that the percentage of TUNEL-positive nuclei was not significantly different between the control and the supplemented group (Fig. 5A, B) (control: 0.40% ± 0.057%; biotin-supplemented 0.35% ± 0.049%). We also determined the expression of the apoptosis protein caspase-3 (Fig. 5C, D). The data showed that the protein abundance of the active form of caspase (caspase-3) was increased in the biotin-supplemented group (control: 0.25 ± 0.030; biotin-supplemented: 0.68 ± 0.12; $P < .005$). No significant difference was observed in the caspase inactive form (procaspase) protein levels (control: 0.67 ± 0.084; biotin-supplemented: 0.84 ± 0.10).

Effects of biotin supplementation on sperm number and quality

The analysis of the effects of biotin supplementation on spermatozoa number found no significant differences in the cauda sperm count between groups (Fig. 6A). The sperm motility of the mice fed the biotin-supplemented diet was significantly decreased compared to the control group (Fig. 6B). Furthermore, sperm from the biotin-supplemented group showed altered morphology (Fig. 6C) with a significant increase in the number of spermatozoa with twisted flagella in the middle piece (control: 6.50 ± 0.68; biotin-supplemented: 30.4 ± 2.04; $P < .0001$) (Fig. 6D).

DISCUSSION

Biotin administration is considered harmless.[28] However, recent studies showing that biotin supplementation affects tissue morphology without modifying toxicity markers have raised concern about the functional consequences of these changes and the safety of pharmacological concentrations of biotin.

Our present results showed that biotin supplementation produced striking effects on the histology of mouse testis and also affects the sperm quality. Compared to the control group, the testes of supplemented mice presented increases in (1) the number of seminiferous tubules with nonvisible lumen; (2) intratubular spaces; (3) sloughing of round germs in the tubular lumen; and (4) increased Leydig cell accumulation. Also, the supplemented group presented alteration in the germinal epithelium and elongated seminiferous tubules with cellular disorganization as well as increased spermatogonia layers. Investigation of the mechanisms involved in these changes revealed increased spermatogonia proliferation. The increased proliferation without augmented spermatozoa number and reduced Jonhsen index suggest delayed spermatogenesis.

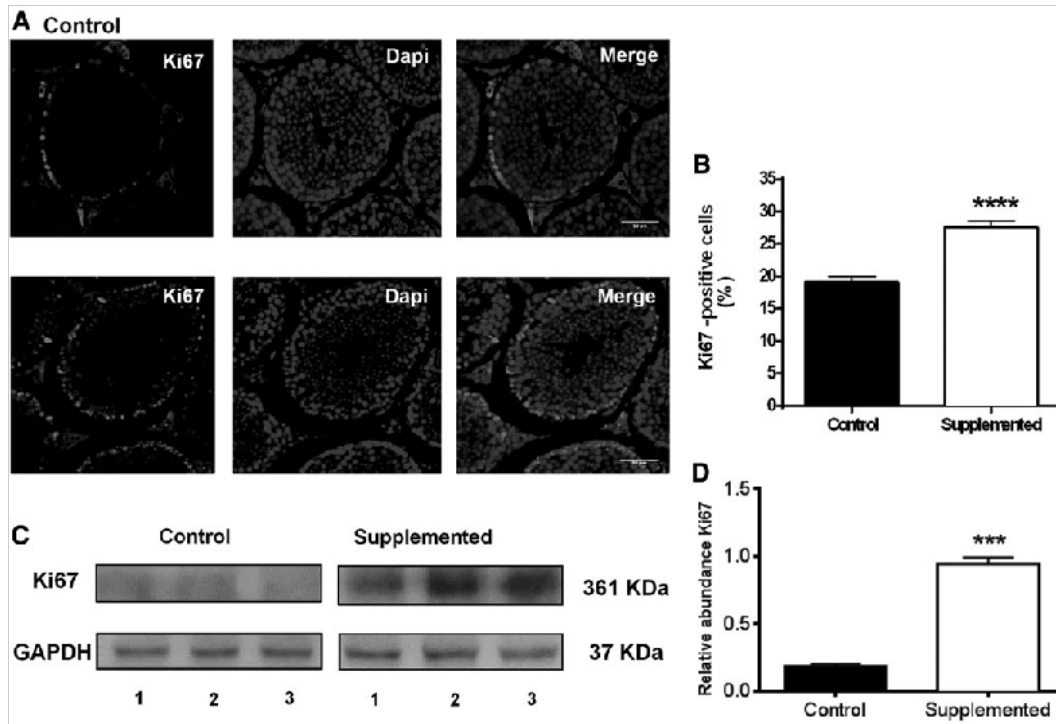

FIG. 4. Effect of biotin supplementation on seminiferous tubule proliferation. **(A)** Images of Ki67-positive nuclei in seminiferous tubules: Ki67 (*red*), and DAPI (*blue*). *Upper panel*: control; *lower panel*: biotin-supplemented group. Scale bar: 50 μm. **(B)** Percentage of seminiferous tubules staining for antigen Ki67 in the nuclei. *Black bar*: control group; *white bar*: biotin-supplemented group. Values represent the mean ± SEM of eight mice from each group. $^{****}P < .0001$. **(C)** Representative western blot analysis of Ki67 and GAPDH. **(D)** Quantification of Ki67 normalized to GAPDH. Values represent the mean ± SEM of eight mice from each group. $^{***}P < .005$.

The changes in the morphology of the seminiferous tubules resulted in defective spermatozoa formation and impaired sperm motility. Some features observed in the biotin-supplemented mice, such as Leydig cell hyperplasia or tubule elongation are produced by other toxics, pathologies[29] or by transgenesis of the plus end-tracking protein EB1,[19] but the ensemble of biotin-induced changes appear to be sui generis; however, we cannot rule out the possibility that these changes might lead to other well-defined degenerative process.

Sawamura *et al.*[11,12] studied the effects of 6 weeks of biotin-supplemented diets on the morphology rat testes. As in our present investigation, they observed no changes in testis weight, food intake, and body weight gain when comparing a diet containing a biotin concentration and the diet used in this work (about 100 mg/kg diet); unfortunately, they did not perform morphological analysis in these animals. In rats fed a biotin-supplemented diet with the 10,000 mg biotin/kg diet, their investigations found that the weight of the testes decreased by about 75%. Histological analysis showed a severe decrease in the total sperm count (less than 1 sperm/mL), increased incidence of sperm with abnormal morphology, mainly round heads, decreased spermatogonia, decreased diameters of seminiferous tubules, and did not showed changes in tubule circularity. No changes were observed in testicular testosterone. In contrast, in our studies in mice fed a biotin-supplemented diet containing 97.7 mg biotin/kg diet over 8 weeks, we found no significant differences in the total sperm count, sperm abnormalities were observed in the flagellum, and the tubule area and the number of spermatogonia layers were increased. Serum testosterone levels were not significantly different when comparing the control and supplemented groups.

It is important to note that the data reported by Sawamura *et al.*[11] are difficult to interpret since the same report showed that rats fed with a diet containing 10,000 mg biotin/kg diet also presented significant decreases in food intake as well as in body, liver, kidney, and brain weight. Indeed, this magnitude of biotin was shown to be toxic in other studies.[17] In contrast, in the conditions of our work (97.7 mg biotin/kg diet during 8 weeks), a model in which unfavorable effects have not been observed on body weight gain, tissues weight, food consumption, external mice appearance, or toxicity

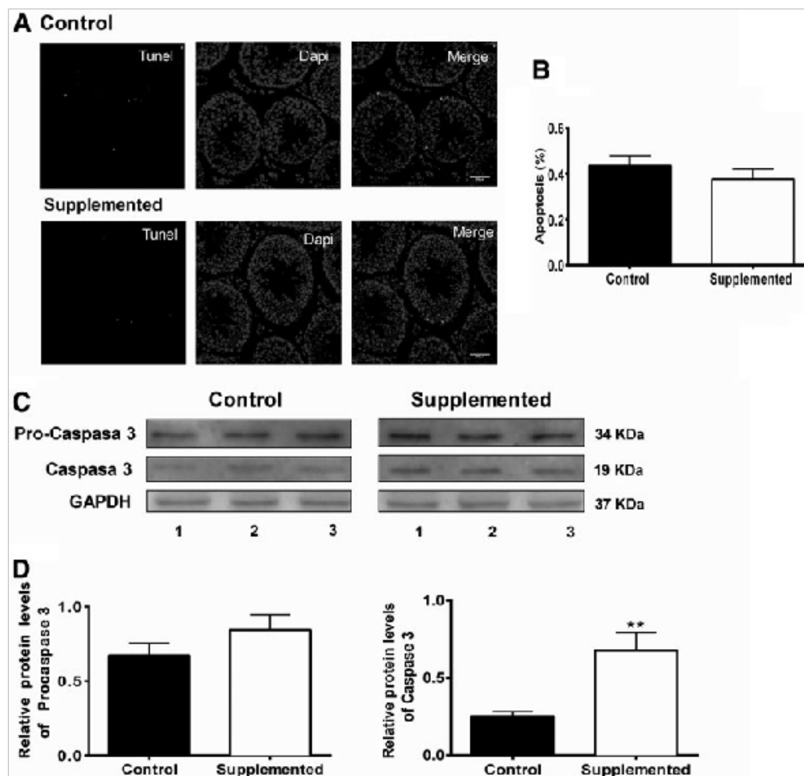

FIG. 5. Effect of biotin supplementation on seminiferous tubule apoptosis. **(A)** Images of TUNEL-positive cells (*green*) and DAPI (*blue*) in seminiferous tubules. *Upper panel*: control; *lower panel*: biotin-supplemented group. Scale bar: 50 μm. **(B)** Percentage of nuclei TUNEL-positive cells in the seminiferous tubules. **(C)** Representative western blot analysis of procaspase-3, caspase-3, and GAPDH. **(D)** Quantification of procaspase-3 and caspase-3 normalized to GAPDH. Values represent the mean ± SEM of eight mice from each group. $^{**}P < .005$.

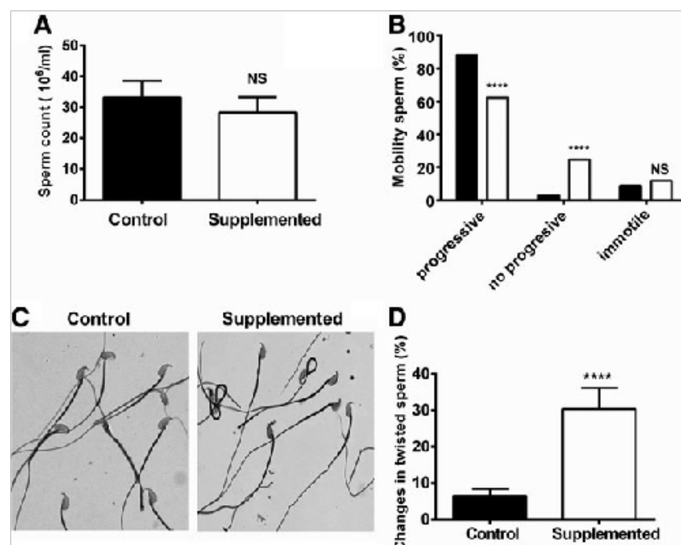

FIG. 6. Effect of biotin supplementation on spermatozoa. **(A)** Number of sperm per mL. *Black bar*: control group; *white bar*: biotin supplemented group. Values represent the mean ± SEM of eight mice from each group. NS, no statistical significance. **(B)** Sperm motility. Values represent the mean percentages ± SEM. $n = 8$ mice per group. $^{****}P < .0001$. **(C)** Representative image of sperm morphology. **(D)** Percentage of changes in twisted spermatozoa. Values represent the mean ± SEM of eight mice from each group. $^{****}P < .0001$.

markers,[5,8,9] we found increased incidence of sperm with abnormal morphology and structural changes of the testes, indicating that the effects of biotin supplementation observed on testes in our experimental model was not due to systemic toxicity.

In prior investigations, we found that normal mice fed the biotin-supplemented diet used in this study presented morphological variations in different organs but did not show function alterations.[5,8,9] The cell topology changes produced in the pancreatic islets did not negatively affect their hormone secretion.[8] In the liver, despite the structural changes, hepatic functions such as gluconeogenesis and glycogenesis were not different from those presented in the control mice.[30] Furthermore, hepatic damage enzymes indicators or oxidative stress markers were not modified.[5] In contrast, in the testes, biotin supplementation, in addition to modifying the tissue morphology, affected their function of sperm quality production. Since testes are very sensitive to toxics[13,14,31] and they have high capacity of biotin accumulation,[11] testes are likely to be one of the tissues more susceptible to pharmacological concentrations of biotin, as demonstrated in the present work.

Proliferation and apoptosis are cellular mechanisms that participate in the normal mammalian testes during spermatogenesis.[32] Proliferation is required to augment cell production, and appropriated apoptosis is a regular cellular mechanism in the normal mammalian testes during the development of spermatogonia and is used to remove excess germ cells. Our results demonstrate that biotin supplementation significantly increases Ki67 in total cellular extracts of testes, which results from the presence of the protein in the spermatogonia and Leydig cells, as shown in the immunofluorescent studies. No significant differences were observed in TUNEL staining. These data suggest that the mechanism involved in enhanced cell layer number is mainly due to cell proliferation. In support of the effects of biotin supplementation on cell proliferation, previous investigations in our laboratory have also found that pharmacological concentrations of biotin increases cell proliferation but not apoptosis.[23]

Biotin supplementation induced Leydig cell hyperplasia; however, at 8 weeks of biotin supplementation, Ki67 labeling was mainly present in the cytoplasm. Leydig cell proliferation is achieved between postnatal days 21 and 35, and they gradually mature into fully steroidogenic adult cells by postnatal day 56.[33,34] Because we initiated biotin-supplementation at 3 weeks of age, the results suggest that Leydig cell hyperplasia was produced between the first and third week of experimentation (3–5 weeks of age), during the time that these cells proliferate.

The changes produced on Leydig cell hyperplasia were not translated into increases in serum testosterone levels. Studies by Sawuamura[11] found that testicular testosterone was not affected by a diet containing 10,000 mg biotin/kg. Further studies will be required to determine the effect of biotin on male steroidogenesis because, in female mice, we found that the biotin-supplemented diet used in the present study increased serum estradiol levels.[9]

It is noteworthy that we found a discrepancy between the increase in the expression of caspase-3 and no difference in TUNEL immunoreactivity. Apoptosis is characterized by nuclear chromatin condensation and DNA fragmentation caused by apoptotic signaling cascades. Apoptosis signaling cascades are mediated by caspases, which trigger cell death by cleaving specific proteins in the cytoplasm and nucleus. Cleaved caspase-3 is an apoptosis effector that translocates to the nucleus, where it cleaves substrates that induce DNA fragmentation. Once activated, caspase-3 might be subject to inhibition by apoptosis protein inhibitors (IAP protein families).[35] Because the TUNEL assay identifies DNA fragmentation, which is the end-stage apoptosis, and caspase-3 is an undergoing apoptosis effector, it is possible that an increased length of a biotin-supplemented diet and/or higher biotin content could translate to increased TUNEL positive cells. However, we cannot rule out the possibility that active caspase-3 might be subject to downstream regulation by apoptosis protein inhibitors.[35] Studies in our laboratory are addressing this issue.

Studies regarding human spermatozoa by Kalthur *et al.*[36] found that *in vitro*, biotin supplementation 2.44 mg/mL (10 nM) augmented motility and prolonged the survival of semen samples. Furthermore, in a recent report,[37] the same group reported that this concentration of biotin increases the fertilizing ability of mice spermatozoa and subsequent mice preimplantation embryo development, suggesting that the vitamin may have benefits in assisted reproduction. In contrast, our present studies show that *in vivo* a biotin-supplemented diet decreased spermatozoa motility. The dissimilar effect of biotin in these studies versus our investigation might be related to the concentration of biotin. The serum concentrations of biotin in mice fed with the present diet for 8 weeks are 144.1 ± 1.59 mg/mL (590 ± 6.5 nM),[8] about 60-fold the amount present in the media of Kalthur studies. However, we cannot rule out that the different study conditions and/or the different sensitivity to biotin between species, as demonstrated in other studies,[38] might account for the discrepancy.

The use of vitamin supplements in larger amounts has escalated considerably in recent years. Water-soluble vitamins are considered to be eliminated and to be safe at higher doses, it is clear that there is a need to assess the risk of vitamins in the context of the wide availability of supplements. Commercially available biotin supplements contain up to 10 mg of the vitamin. In humans, supplementation of 1.2 mg biotin/day for 14 days results in serum concentrations in a range of 9.4–47.7 nM[39] (2.29–11.6 mg/mL), so it is likely that the serum biotin levels attained by supplements with high levels of biotin could reach the vitamin concentration in the same order of magnitude observed in our studies.[8]

Classical toxicity tests[40,41] and other studies[7] have suggested that biotin administration is harmless[28]; however, taking into account the effects of biotin on testes function and morphology obtained in this work, and that biotin supplements with high levels of biotin could attain the vitamin concentrations observed in our studies,[8] human

pharmacokinetic and pharmacodynamic studies are imperative given that biotin's UL has not been established.[3,4]

In conclusion, the results of this work showed that studies meant to determine the safety of pharmacological concentrations of biotin need to consider its effects on tissue morphology and function.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interest exists.

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