UNIVERSIDADE FEDERAL DE MINAS GERAIS

Instituto de Ciências Biológicas

Matheus Felipe Fonseca Gonçalves

Tese de Doutorado

AVALIAÇÃO DA FUNÇÃO DOS GENES *GATA-1* E *SRY* NA FISIOLOGIA TESTICULAR DE CAMUNDONGOS ADULTOS

BELO HORIZONTE

Novembro de 2021

Matheus Felipe Fonseca Gonçalves

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Tese de doutorado apresentada ao Programa de Pós-graduação em Biologia Celular, Departamento de Morfologia, do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais como requisito para a obtenção do título de doutor em Biologia Celular.

Orientador: Guilherme Mattos Jardim Costa

Coorientadora: Samyra Maria dos Santos Nassif Lacerda

Laboratório de Biologia Celular

Departamento de Morfologia-ICB/UFMG

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ATA DA DEFESA DE TESE DE DOUTORADO DE MATHEUS FELIPE FONSECA GONÇALVES

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Às **quatorze horas** do dia **30 de novembro de 2021**, reuniu-se, por meio de plataforma digital, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"AVALIAÇÃO DA FUNÇÃO DOS GENES GATA-1 E SRY NA FISIOLOGIA DE CAMUNDONGOS ADULTOS"**, requisito final para obtenção do grau de Doutor em Biologia Celular. Abrindo a sessão, o Presidente da Comissão, **Dr. Guilherme Mattos Jardim Costa**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa do candidato. Logo após, a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	**Instituição**
Dr. Guilherme Mattos Jardim Costa	UFMG
Dra. Samyra Maria dos Santos Nassif Lacerda	UFMG
Dr. Antônio Carlos Santana Castro	UFJF
Dr. André Lucas Caldeira-Brant	University of Pittsburgh School of Medicine
Dr. Marcos de Lucca Moreira Gomes	UFTM
Dr. Lucas Miranda Kangussu	UFMG
Dra. Lídia Maria de Andrade	UFMG

Pelas indicações, o candidato foi considerado: APROVADO.

O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 30 de novembro de 2021.**

Dr. Guilherme Mattos Jardim Costa (Orientador)

Drª. Samyra Maria dos Santos Nassif Lacerda (Coorientadora)

Dr. Antônio Carlos Santana Castro

Dr. André Lucas Caldeira-Brant

Dr. Marcos de Lucca Moreira Gomes

Dr. Lucas Miranda Kangussu

Dra. Lídia Maria de Andrade

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Dedico este trabalho aos meus pais

que, mesmo nas vitórias ou nas derrotas,

estão sempre ao meu lado me dando força e foco

para continuamente ser uma pessoa melhor.

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- Núcleo de Fisiologia Genômica e Funcional (NUFIGEN) do Departamento de Fisiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais (UFMG);

- Angiogênese e células tronco do Departamento de Fisiologia e Farmacologia do Instituto de Ciências Biológicas (ICB) da Universidade Federal de Minas Gerais (UFMG);

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- Laboratório de sinalização cardíaca, Departmento de Fisiologia e Biofísica, Instituto de Çiencias Biológicas (ICB) da Universidade Federal de Minas Gerais (UFMG).

- Centro para pesquisa e desenvolvimento de fármacos, Departmento de Morfologia, Instituto de Çiencias Biológicas (ICB) da Universidade Federal de Minas Gerais (UFMG).

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“Tenho a impressão de ter sido uma criança

brincando à beira-mar,

divertindo-me em descobrir uma pedrinha mais lisa

ou uma concha mais bonita que as outras,

enquanto o imenso oceano da verdade

continua misterioso diante de meus olhos”. (Isaac Newton)

Resumo

O presente trabalho se propôs a analisar a função de dois genes, Gata-1 e Sry, expressos no testículo. Gata-1 é um fator de transcrição da família GATA que foi inicialmente identificado como um regulador crucial da diferenciação de células hematopoiéticas, mas sabe-se agora que a expressão deste gene não se limita a este sistema. Embora o testículo também seja um local de expressão significativa de Gata-1, seu papel na espermatogênese permanecia amplamente inexplorado. Embora Sry seja bem conhecido por desempenhar um papel na determinação do sexo masculino durante o estágio embrionário dos mamíferos, seu papel na função dos testículos do animal adulto não foi bem estudado. Nesse contexto, avaliamos a morfofisiologia testicular de camundongos ΔdblGATA adultos com mutação na proteína GATA-1 e tratamos camundongos machos adultos selvagens com siRNA, promovendo o silenciamento do RNA mensageiro do gene Sry. Os achados mostraram que o gene Gata-1 estava mais intimamente associado ao compartimento intertubular. Os animais mutantes Gata-1 apresentaram alterações morfofuncionais nas células de Leydig, incluindo diminuição de volume, alteração na expressão de enzimas esteroidogênicas e diminuição dos níveis de testosterona. Em relação ao Sry, os animais tratados apresentaram alterações significativas na vascularização testicular e no compartimento tubular, com liberação maciça e apoptose de células germinativas do epitélio seminífero. Como resultado, nossa pesquisa identificou genes que desempenham um papel significativo nas funções endócrinas e exócrinas do testículo. Mostramos que a proteína GATA-1 é um fator crítico para a atividade esteroidogênica no testículo, enquanto a expressão de Sry é essencial para a produção de gametas em animais adultos.

Palavras-chave: *Sry*, siRNA, espermatogênese, camundongo, células de Leydig, testículo, ΔdblGATA, testosterona.

ABSTRACT

The present work proposed to analyze the function of two genes, Gata-1 and Sry, expressed in the testis. Gata-1 is a transcription factor of the GATA family that was initially identified as a crucial regulator of hematopoietic cell differentiation, but it is now known that the expression of this gene is not limited to this system. Although the testis is also a site of significant Gata-1 expression, its role in spermatogenesis remained largely unexplored. Although Sry is well known to play a role in determining the male sex during the mammalian embryonic stage, its role in the function of the adult animal's testicles has not been well studied. In this context, we evaluated the testicular morphophysiology of adult ΔdblGATA mice with a mutation in the GATA-1 protein, and we treated wild adult male mice with siRNA, promoting the silencing of Sry messenger RNA. The findings showed that the Gata-1 gene was more closely associated with the intertubular compartment. The Gata-1 mutant animals displayed morphofunctional changes in the Leydig cells, including a decrease in volume, a change in the expression of steroidogenic enzymes, and a decrease in testosterone levels. Concerning Sry, treated animals showed significant changes in testicular vascularization and the tubular compartment, with massive release and apoptosis of germ cells from the seminiferous epithelium. As a result, our research identified genes that play a significant role in the endocrine and exocrine functions of the testis. We showed that the GATA-1 protein is a critical factor for steroidogenic activity in the testis, while the Sry expression is essential for gamete production in adult animals.

Keywords: *Sry*, siRNA, spermatogenesis, mice, Leydig cell, testis, ΔdblGATA, testosterone.

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Ao longo do texto, quando os símbolos estiverem escritos em caixa alta (maiúsculo) estamos nos referindo a proteína e quando somente a primeira letra estiver em maiúsculo e o restante em itálico fazemos menção ao gene ou do RNA.

3BHSD: do inglês, 3beta-hydroxysteroid dehydrogenase

Ar: receptor de andrógeno

CASPASE-3: do inglês, cysteine-aspartic-acid-proteases-3

Cyp11: do inglês, Cytochrome P450, family 11

DAB: Diaminobenzidina

Dmrt1: do inglês, Doublesex and mab-3 related transcription factor 1

Eif2s3x: do inglês, Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked

GATA-1: GATA-binding factor 1

GFRA-1: do inglês, GDNF family receptor alpha-1

GSI: do inglês, índice gonadossomático

Insl3: Insulin-Like Peptide 3

Ntc: Nanotubos de carbono

qPCR: Reação em cadeia da polimerase em tempo real ou quantitativa

RNAm: RNA mensageiro

RT-PCR: Reação em cadeia da polimerase via transcriptase reversa

Sf1: do inglês, Splicing factor 1

siRNA: RNA de interferência

Sox9: do inglês, SRY-related HMG-box

Sry: do inglês, sex-determining region Y

Star: do inglês, steroidogenic acute regulatory protein

TUNEL: do inglês, Terminal deoxynucleotidyl transferase dUTP nick end labeling

Vegf: Fator de crescimento do endotélio vascular

SUMÁRIO

INTRODUÇÃO E REVISÃO DE LITERATURA

1.1 Estrutura testicular

O testículo é um órgão com funções exócrina e endócrina, envolvido por uma espessa cápsula de tecido conjuntivo, a túnica albugínea. Esta túnica, de maneira variada nas diferentes espécies de mamíferos, envia trabéculas de tecido conjuntivo, os septos testiculares, para o interior do órgão até a região do mediastino testicular, dividindo o testículo em lóbulos (Russell et al., 1990; Hess & França, 2007; Wu et al., 2019). Funcionalmente, o testículo dos mamíferos pode ser dividido em dois compartimentos principais: o compartimento intersticial, também chamado de espaço intertubular, e o compartimento tubular ou de túbulos seminíferos (Russell et al., 1990; Hess & França, 2007; Wu et al., 2019).

Os elementos componentes do compartimento intertubular são as células de Leydig, vasos sanguíneos e linfáticos, nervos e uma população celular variável constituída principalmente de fibroblastos, macrófagos, linfócitos, células dendríticas e mastócitos (Russell et al., 1990; Lara et al., 2018). O compartimento dos túbulos seminíferos constitui a maior parte do testículo, ocupando, na grande maioria dos mamíferos, de 70% a 90% do parênquima testicular (França & Russell, 1998). Os túbulos seminíferos são constituídos por túnica própria, epitélio seminífero e lúmen tubular. A túnica própria reveste o túbulo externamente, sendo composta de células peritubulares mióides e matriz extracelular, enquanto o epitélio seminífero é composto pelas células germinativas e de Sertoli (Russell et al., 1990; Hess & França, 2007; Wu et al., 2019).

1.2 Espermatogênese em mamíferos

A espermatogênese é um processo cíclico, altamente complexo e organizado que ocorre nos túbulos seminíferos de animais sexualmente maduros (França & Russell, 1998; França et al., 1998; Godinho, 1999; Almeida, 2002). Baseado em características morfológicas e funcionais, o processo espermatogênico pode ser dividido em três fases: (a) fase proliferativa ou espermatogonial, caracterizada por várias e sucessivas divisões mitóticas dos diferentes tipos de espermatogônias; (b) fase meiótica ou espermatocitária, na qual ocorre a duplicação do DNA, a recombinação gênica e duas divisões meióticas (reducional e equacional) que resultam na formação de células haplóides denominadas espermátides; e (c) fase de diferenciação ou espermiogênica, onde as espermátides arredondadas passam por drásticas alterações morfológicas e funcionais tais como formação do acrossoma, do flagelo e condensação nuclear, resultando numa célula altamente especializada, o espermatozoide, o qual estará apto para o processo de capacitação e fertilização (Russell et al., 1990; Sharpe, 1994; Hess & França, 2007; Wu et al., 2019).

1.2.1 Fase espermatogonial

Na fase espermatogonial ou proliferativa, as espermatogônias tronco se dividem originando células semelhantes (autorenovação) ou células germinativas comprometidas com a formação de espermatozoides (diferenciação). As espermatogônias tronco fazem parte de uma subpopulação denominadas de espermatogônias indiferenciadas, compostas pelas espermatogônias Aisoladas, Apareadas e Aalinhadas (revisado por de Rooij, 2017). A outra subpopulação é denominada de espermatogônias diferenciadas, sendo compostas pelas espermatogônias A1, A2, A3, A4, Intermediárias e do tipo B. Trabalhos fundamentados em características morfológicas sugerem que as espermatogônias indiferenciadas possuem localização preferencial (nicho) nos túbulos seminíferos de mamíferos (Chiarini-Garcia et al., 2001; Chiarini-Garcia et al., 2003; Nascimento et al., 2009; Campos-Junior et al., 2012; Costa et al., 2012). Resultados semelhantes foram obtidos utilizando-se diversas técnicas, tais como o transplante de células germinativas, as quais demonstraram que as espermatogônias indiferenciadas se localizam preferencialmente em regiões dos túbulos seminíferos adjacentes aos vasos sanguíneos (nicho) e, à medida que se diferenciam, as células resultantes se afastam dessa região do túbulo (Yoshida et al., 2007).

1.2.2 Fase espermatocitária

As espermatogônias B sofrem a última divisão mitótica e originam os espermatócitos primários conhecidos como pré-leptóteno. Estas células representam o começo da prófase meiótica (Hess & França, 2007; Wu et al., 2019). A prófase da primeira divisão meiótica é longa e subdividida em cinco estágios denominados de pré-leptóteno, leptóteno, zigóteno, paquíteno e diplóteno. No estágio inicial, os espermatócitos primários em pré-leptóteno realizam a última síntese de DNA nas células germinativas. No estágio seguinte, os espermatócitos em leptóteno apresentam aumento do volume celular e distribuição homogênea de cromatina no núcleo. Nos espermatócitos em zigóteno ocorre espessamento cromossômico, início de pareamento dos cromossomos homólogos e formação do complexo sinaptonêmico, sendo este último visível somente sob microscopia eletrônica de transmissão (Billard, 1986; Grier & Neidig, 2000, Nóbrega, 2006). Este complexo persiste até o final do estágio de paquíteno que é a fase mais longa da prófase meiótica, na qual ocorre a recombinação e a segregação gênica, importante para a diversidade de indivíduos da mesma espécie. Os cromossomos, nessa fase, apresentam-se completamente pareados e compactos. Durante a Meiose I, os espermatócitos em paquítenos também aumentam de tamanho antes de se transformarem em diplótenos que se dividem para formar os espermatócitos secundários (células n duplo). Essas células, caracterizadas pela morfologia arredondada, citoplasma escasso e núcleo com cromatina condensada, passam rapidamente por uma segunda divisão meiótica (Meiose II) para produzir células

haplóides (células n simples), denominadas de espermátides (Russell et al., 1990; Hess & França, 2007; Wu et al., 2019).

1.2.3 Fase espermiogênica

A fase que corresponde à transformação de espermátides arredondadas em espermátides alongadas, maturação e espermiação dos espermatozoides no lúmen do túbulo seminífero é conhecida como fase espermiogênica ou de diferenciação (Hess & França, 2007). Baseado no sistema de desenvolvimento do acrossoma, esta fase de diferenciação das espermátides requer pelo menos 4 etapas conhecidas como: fase de Golgi, fase dos grânulos acrossomais (Capuz), fase acrossomal e fase de maturação (Russel et al., 1990; Hess & França, 2007; Wu et al., 2019). Nos túbulos seminíferos, as células germinativas não estão organizadas ao acaso e sim em associações celulares características denominadas de estádios, os quais se sucedem de maneira bastante ordenada ao longo do tempo, formando o ciclo do epitélio seminífero.

1.3 Células de Sertoli

As células de Sertoli, através de junções de oclusão, dividem o epitélio seminífero em dois compartimentos, o compartimento basal onde se localizam as espermatogônias e os espermatócitos primários na fase inicial da prófase meiótica (pré-leptótenos e leptótenos), e o compartimento adluminal, no qual encontram-se os espermatócitos primários a partir da fase de zigóteno, espermatócitos secundários e espermátides. Desta forma, o compartimento adluminal está totalmente sob o controle das células de Sertoli (barreira de célula de Sertoli), que propicia um microambiente isolado e imunoprivilegiado essencial para o desenvolvimento do processo espermatogênico (Russell et al., 1990; Fijak & Meinhardt, 2006; Mazaud-Guittot et al., 2010; Lara et al., 2018). No lúmen tubular encontram-se o fluido secretado pelas células de Sertoli e os espermatozoides recém-espermiados.

Durante o processo espermatogênico, as células de Sertoli e as células germinativas interagem de maneira bastante complexa, tanto física quanto bioquimicamente. Existem diversas formas de junções intercelulares entre estes dois tipos celulares, incluindo-se os desmossomos, junções do tipo “gap”, junções à base de actina, conhecidas como especializações ectoplasmáticas, e complexos túbulos-bulbares (Lara et al., 2018). Apesar de serem postuladas várias funções para estes dispositivos juncionais, existem ainda poucas evidências experimentais para sustentar o papel preciso dos mesmos (Russell & Griswold, 1993; Mazaud-Guittot et al., 2010). No entanto, fica bastante evidente a necessidade da interação das células germinativas com os componentes somáticos do testículo, principalmente células de Sertoli, Leydig e peritubulares mióides, para que o processo espermatogênico transcorra de maneira normal e eficiente (Russell et al., 1994; França & Russell, 1998; Welsh et al., 2009; França

et al., 2016). A integridade funcional da membrana basal elaborada pelas células de Sertoli e peritubulares mióides é também essencial para o processo espermatogênico (Dym, 1994).

Além da formação da barreira hemato testicular já comentada anteriormente, as células de Sertoli desempenham outras funções essenciais para o desenvolvimento das células germinativas. Assim, podem ser citadas o fornecimento de nutrientes e inúmeros outros fatores importantes para as células germinativas, mediação da ação do FSH e da testosterona, fornecimento de suporte físico (sustentação) para as células espermatogênicas, participação ativa no processo de liberação (espermiação) das espermátides para o lúmen tubular, fagocitose do excesso de citoplasma (corpos residuais) resultante da liberação das células espermiadas e fagocitose de células germinativas que sofrem apoptose. As células de Sertoli secretam ainda fluido em direção ao lúmen tubular, o qual possui substâncias importantes para a função epididimária e maturação espermática, servindo também de veículo para o transporte dos espermatozoides (França et al., 2016; Lara et al., 2018). A secreção de fluido também ocorre em direção ao compartimento intertubular, estando a mesma envolvida com os mecanismos de regulação parácrina de outros tipos celulares do testículo tais como as células peritubulares mióides, Leydig, do sistema imune e musculares lisas dos vasos (Russell & Griswold, 1993; Sharpe, 1994; França & Russell, 1998; Hess & França, 2007; Wu et al., 2019).

1.4 Células de Leydig

As células de Leydig são bastante conhecidas por sua marcante produção de andrógenos, os quais são sintetizados a partir de uma molécula base, o colesterol (Bardin, 1996). Esta produção de andrógenos ocorre através de estímulos do LH (hormônio luteinizante) em receptores localizados na membrana citoplasmática das células de Leydig. À semelhança do FSH, o LH é uma glicoproteína sintetizada e secretada na adenohipófise sob a influência do hormônio liberador de gonadotrofinas (GnRH) proveniente do hipotálamo. O controle “feedback” negativo do LH é exercido pela testosterona tanto na adenohipófise quanto no hipotálamo (Shupnike & Schreihofer, 1997; Corradi et al., 2016). Nos testículos, existem receptores para andrógenos nas células de Sertoli, células peritubulares mióides, musculares lisas dos vasos e na própria célula de Leydig (Suárez-Quian et al., 1999; Lara et al., 2018). Dentre os andrógenos sintetizados pelas células de Leydig incluem-se a testosterona e a diidrotestosterona, os quais são responsáveis pela diferenciação do aparelho genital masculino e da genitália externa na fase fetal (Pelleniemi et al., 1996) e pelo aparecimento dos caracteres sexuais secundários e a manutenção quantitativa da espermatogênese a partir da puberdade (Sharpe, 1994; Zirkin et al., 1994; De Gendt et al., 2004; Lara et al., 2018). Particularmente, a diidrotestosterona é responsável pela manutenção funcional das glândulas sexuais acessórias e do epidídimo (Fan & Robaire, 1998; Goyal et al., 1999).

1.5 Quantificação da espermatogênese

A apoptose de células germinativas é parte integral da espermatogênese, ocorrendo normalmente em várias fases deste processo (Roosen-Runge, 1977; Lara et al., 2018). Apoptoses representam papel muito importante para a homeostase da espermatogênese, refletindo diretamente na produção espermática diária característica de cada espécie (Clermont, 1972; Sharpe, 1994). Embora apoptoses sejam comumente observadas durante a fase de divisões meióticas, principalmente devido a danos cromossomais (Roosen-Runge, 1977; Selva et al., 2000; Xu et al., 2003), a regulação do número de células germinativas ocorre, de forma mais prevalente, durante a fase espermatogonial (De Rooij & Lok, 1987), fazendo com que um número de células espermatogênicas por células de Sertoli seja adequado (França & Russell, 1998; Lara et al., 2018).

O estudo quantitativo das células que compõem o epitélio seminífero, é importante para um entendimento mais completo da espermatogênese e de como a estrutura testicular se comporta em condições experimentais e patológicas (Roosen-Runge & Giesel Jr., 1950; Wing & Christensen, 1982; França, 1991; França & Russell, 1998). A obtenção da proporção volumétrica (%) entre os diversos componentes do testículo e do epitélio seminífero, bem como a estimativa do tamanho e número de células de Leydig por testículo, fornecem também importantes dados para se avaliar a função testicular (França, 1991; Silva Jr., 2000).

Estudos recentes mostram que o número de células de Sertoli por testículo é o principal fator na determinação da produção espermática e do tamanho do testículo (Orth et al., 1988; Hess et al., 1993; França et al., 1995; Rocha et al., 1999; Silva Jr., 2000; Miranda, 2002; França et al., 2016). Tal pressuposto baseia-se no fato de que as células de Sertoli têm uma capacidade de suporte de células germinativas relativamente fixa para cada espécie, e que a população deste tipo celular se mantém estável após a puberdade (Orth, 1982; Orth et al., 1988; França & Russell, 1998), não havendo alteração no seu número ao longo dos estádios do ciclo do epitélio (Roosen-Runge & Giesel jr., 1950; Wing & Christensen, 1982; França, 1991; Neves, 2001). Desta forma, o número de células germinativas suportadas por uma única célula de Sertoli é a melhor indicação da eficiência funcional da célula de Sertoli e da produção espermática (Russell & Peterson, 1984; Sinha-Hikim et al., 1989; França & Russell, 1998).

1.6 Fatores de transcrição envolvidos com a espermatogênese

Fatores de transcrição são proteínas reguladoras que se ligam a sequências específicas de DNA nas regiões reguladoras ou promotoras dos genes-alvo. Eles estão envolvidos na expressão gênica basal e específica do tecido. Existem várias classes de fatores de transcrição que foram definidas com base em similaridades na estrutura dos respectivos domínios de ligação ao DNA (Viger et al., 2004). A diferenciação das células germinativas masculinas, que culmina na produção dos espermatozóides funcionais, são regulados por fatores transcricionais e pós-

transcricionais. No presente estudo, abordaremos dois fatores de transcrição que foram pouco explorados na homeostase testicular de camundongos adultos: GATA-1 e SRY.

1.6.1 GATA-1

A família GATA de fatores de transcrição apresenta dedos de zinco para ligação ao DNA, mais especificamente na sequência nucleotídica (A / TGATAA / G). Eles foram originalmente identificados como reguladores cruciais do desenvolvimento do coração, da diferenciação das células sanguíneas e das células do sistema imunológico. Sabe-se, entretanto, que a expressão dos genes GATA não se limita a esses dois sistemas. O testículo e ovário também são locais importantes da expressão do GATA. Atualmente, os fatores GATA já foram descritos no desenvolvimento gonadal, determinação e diferenciação sexual masculina e esteroidogênese (Viger et al., 2004). O primeiro fator de transcrição, que se ligava especificamente aos elementos cis GATA, foi clonado a partir de células eritróides, sendo denominado GATA-1 (Tsai et al, 1989). Também foi demonstrado que o GATA-1 continha um domínio de ligação ao DNA que consistia em dois dedos de zinco semelhantes (Tsai et al., 1989; Weiss e Orkin, 1995a).

A partir do GATA-1, cinco fatores de transcrição adicionais com domínios de ligação ao DNA semelhantes, foram identificados em animais vertebrados (denominados GATA-2 a GATA-6) (Weiss e Orkin, 1995a; Molkentin, 2000). Estes fatores podem ser separados em dois subgrupos com base em sua distribuição tecidual: um hematopoiético (GATA-1, GATA-2 e GATA-3) e outro cardíaco (GATA-4, GATA-5, GATA-6) (Viger et al., 2004). Dos seis fatores GATA, quatro são expressos nas gônadas de mamíferos: GATA-1 (Ito et al., 1993; Yomogida et al., 1994; Viger et al., 1998), GATA-2 (Siggers et al., 2002), GATA-4 (Heikinheimo et al. , 1997; Viger et al, 1998; Ketola et al, 1999; McCoard et al., 2001) e GATA6 (Heikinheimo et al., 1997; Ketola et al., 1999; Robert et al., 2002).

Em geral, os fatores GATA são expressos, em sua maioria, nas células somáticas das gônadas. As exceções são GATA-2, expresso em células germinativas do ovário de camundongo (Siggers et al., 2002) e GATA-4, expresso em células germinativas do testículo humano e de camundongos. (Ketola et al., 1999; Ketola et al., 2000). O significado da expressão de GATA-1 nas células testiculares, no entanto, ainda não foi demonstrado. Dessa forma, na presente investigação, avaliamos a função testicular dos animais ΔdblGATA que apresentam uma mutação na proteína GATA-1 para verificar a atividade gametogênica e esteroidogênica desses animais.

1.6.2 SRY

Até 1980, os cientistas sabiam da existência de uma região no cromossomo Y que era crucial para a determinação sexual. Esta região ficou conhecida como TDF (Testis-determining factor), porém somente em 1990, Andrew Sinclair e Jamie Foster descobriram que esta região era na verdade o gene *Sry* (Sinclair et al.,1990). Este gene é um membro da família SOXB do locus Sox, que inclui *Sox1, Sox2, Sox3* e *Sry*. O locus *Sry* (região de determinação sexual no cromossomo Y) foi o primeiro membro da família Sox de fatores transcricionais identificado (Sinclair et al.,1990). *Sox1 e Sox2* são autossômicos na maioria dos mamíferos, e o *Sox3* é ligado ao cromossomo X. Acredita-se que o *Sry* se originou a partir de uma mutação do gene *Sox3* (Sato et al., 2010).

Sabe-se que o *Sox3* e o *Sry* são expressos no testículo do animal adulto (Weiss et al., 2003). O Sry surgiu por volta de 180 a 210 milhões de anos atrás, com o aparecimento dos marsupiais. Nos animais menos derivados, como os mamíferos monotremados, aves e répteis (em que não existia *Sry*), especula-se que outros genes, como o *Sox9, Dmrt1, Sf1 e Wt1* seriam os responsáveis pela diferenciação testicular (Wallis et al., 2007). A descoberta do gene Sry, responsável pela diferenciação da crista genital bipotencial em testículo, representou um grande marco na ciência. Seu papel foi evidenciado pelo desenvolvimento de testículos em fêmeas de camundongos com a adição do Sry na fase embrionária (Koopman et al., 1991). Além de iniciar a cascata de diferenciação do testículo em embriões, os transcritos do Sry são encontrados em diversos tecidos nos camundongos adultos.

Apesar dos avanços científicos, diversas questões permanecem desconhecidas sobre a regulação fina desse gene durante a diferenciação e maturação do testículo e, principalmente, sobre a função no processo espermatogênico de indivíduos adultos. Dados preliminares do nosso laboratório demonstram que o silenciamento do Sry promove a infertilidade animal com alterações morfológicas severas no testículo em curto período de tempo. A consolidação desses achados certamente fornecerá bases para tratamento de infertilidade masculina. Adicionalmente, em outra vertente, métodos contraceptivos em animais adultos poderão ser desenvolvidos ao se impedir a expressão desse gene de forma específica no testículo.

REFERÊNCIAS BIBLIOGRÁFICAS (PRESENTE NO FINAL DO VOLUME**)**

2. OBJETIVOS

Avaliar a função do gene *Gata-1* e *Sry* na fisiologia testicular de indivíduos adultos.

#1 – GATA-1 mutation alters the spermatogonial phase and steroidogenesis in adult mouse testis

- Comparar a biometria dos animais mutantes com animais controles;
- Determinar a frequência de estádios do ciclo do epitélio seminífero e a duração da espermatogênese por meio da injeção de Brdu;
- Realizar análises morfométricas testiculares, incluindo a proporção volumétrica, diâmetro tubular, altura do epitélio seminífero;
- Mensurar o volume nuclear e número da célula de Sertoli por testículo;
- Realizar a contagem de células germinativas presentes no epitélio seminífero;
- Determinar as razões celulares do epitélio seminífero;
- Determinar a produção espermática diária;
- Mensurar o número e volume das células de Leydig por testículo;
- Investigar marcadores de morte celular e da via esteroidogênica através de imuno-histoquímicas, utilizando os anticorpos anti-CASPASE-3, anti-3BHSD, anti-GATA-4, anti-GFRA-1, anti-GATA-1, anti-17BHSD;
- Avaliar a expressão gênica do *Ar, Star, Cyp11, Cyp17, Cyp19, Hsd3b1, Hsd3b6, Insl3, Wt1 e Gapdh* através da técnica de qPCR.
- Determinar os níveis de testosterona sérica dos animais *in situ* e *in vitro,* e níveis séricos de LH;
- Realizar análise espermática (motilidade, morfologia, de ambos os grupos).

#2 – Testicular function after post-transcriptional silencing of *Sry* gene

- Comparar a biometria dos animais controles e tratados;
- Identificar os nanotubos de carbono no testículo por microscopia eletrônica de transmissão;
- Avaliar o fluxo sanguíneo testicular através da técnica do laser Doppler;
- Avaliar a expressão relativa do gene *Sry* e de genes relacionados, como *Sox9* e *Dmrt1*;
- Avaliar qualitativamente a histologia testicular e epididimária;

- Investigar as proporções volumétricas (%) entre os diferentes componentes do parênquima testicular;
- Mensurar o diâmetro tubular e altura do epitélio seminífero;
- Avaliar o percentual de túbulos que apresentaram alterações nas células germinativas;
- Calcular o número de células de Sertoli por testículo e por grama de testículo;
- Determinar o volume individual das células de Leydig, bem como do número de células de Leydig por testículo e por grama de testículo;
- Avaliar através de imunohistoquímica a expressão das proteínas SRY, SOX9, CASPASE-3, CONEXINA-43;
- Investigar, pela técnica de TUNEL, a presença de células testiculares em apoptose;
- Avaliar a expressão relativa do *Ar*, *Star*, *Vegf*, *Claudina-11* e *Caspase-3;*
- Determinar os níveis séricos de testosterona dos animais.

Artigo 1

GATA-1 mutation alters the spermatogonial phase and steroidogenesis in adult mouse testis

Artigo 1 - GATA-1 mutation alters the spermatogonial phase and steroidogenesis in adult mouse testis

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ABSTRACT

GATA-1 is a transcription factor from the GATA family, which features zinc fingers for DNA binding. This protein was initially identified as a crucial regulator of blood cell differentiation, but it is currently known that the *Gata-1* gene expression is not limited to this system. Although the testis is also a site of significant GATA-1 expression, its role in testicular cells remains considerably unexplored. In the present study, we evaluated the testicular morphophysiology of adult ΔdblGATA mice with a mutation in the GATA-1 protein. Regarding testicular histology, GATA-1 mutant mice exhibited few changes in the seminiferous tubules, such as a higher proportion of differentiated spermatogonia and high number of apoptotic pre-leptotene spermatocytes (Caspase-3-positive). Considering the sperm analyses, a high frequency of sperm head defects was also observed in ΔdblGATA mice. The main differences were observed in the intertubular compartment, as ΔdblGATA mice showed several morphofunctional changes in the Leydig cells, such as reduced volume and alterations in the expression of enzymes that promote androgen biosynthesis, resulting in lower testosterone levels. These results are consistent with phenotypic and biometric observations since the mutant mice presented shorter anogenital distance and reduced accessory sexual gland weight. It was also possible to observe higher levels of LH and hyperplasia of Leydig cells associated with high expression of *Insl3* in ΔdblGATA Leydig cells. In conclusion, our findings suggest that GATA-1 protein is an important factor for the initial steps of spermatogenesis as well as for the steroidogenic activity in the testis.

Keywords: Leydig cell, testis, ΔdblGATA, testosterone, spermatogonia,

1. INTRODUCTION

Proteins of the GATA family are transcription factors that recognize the DNA consensus sequence (T/A) GATA (A/G) (Evans *et al*. 1988; Wall *et al*. 1988; Tsai *et al*. 1989; Martin and Orkin 1990; Tsai *et al*. 1991). GATA-1 was the first described transcription factor featuring zinc finger domains for DNA binding (Tsai *et al*. 1989; Weiss and Orkin 1995a; Kumar *et al*. 2018). After that, five other transcription factors with similar zinc finger domains were identified in vertebrates (named GATA-2 to GATA-6) (Weiss and Orkin 1995a; Molkentin 2000). It is well known that GATA-1 is expressed by erythroid and megakaryocytic blood lineages, playing a crucial role in cell maturation and differentiation (Morceau *et al*. 2004; Lally *et al*. 2019).

In the male genital system, GATA-1, GATA-4 and GATA-6 expressions were identified in prostate and testes (Arceci *et al*. 1993; Ito *et al*. 1993; Grepin *et al*. 1994; Yomogida *et al*. 1994; Onodera *et al*. 1997; Feng *et al*. 1998; Viger *et al*. 1998; Ketola *et al*. 1999; Zhang *et al*. 2019). Testicular expression of GATA-1 is age-dependent in mice Sertoli cells (SC), i.e., its expression occurs at low levels in some SC nuclei at seven days post-partum and becomes more pronounced at nine days post-partum (Yomogida *et al*. 1994). Although GATA-1 is expressed in SCs in all seminiferous tubule (ST) cross-sections at 21 days of age, this pattern does not persist at 35 days of age when some cross-sections no longer express this factor (Yomogida *et al*. 1994). Interestingly, cryptorchid animals, which have a reduced amount of germ cells, express GATA-1 in all cross-sections. Thus, it is suggested that the presence of germ cells may suppress the expression of GATA-1 in SCs after the organ has fully matured (Yomogida *et al*. 1994). Zhang and colleagues (2002) also detected *Gata-1* mRNA in Leydig cells (LCs) of 21-day-old rats. However, additional data is still missing to confirm whether GATA-1 is required for the normal function of LCs *in vivo* (Viger *et al*. 2004).

Although the gene inactivation technique is of great value for a better understanding of a target gene function, this methodology is not feasible for studying GATA transcription factors because knockout animals die during the embryonic phase due to heart failure and problems in hematopoietic precursor cell development (Pevny *et al*. 1991; Kuo *et al*. 1997; Molkentin *et al*. 1997; Morrisey *et al*. 1998; Viger *et al*. 2004). The GATA-1 protein is formed after the testis and hematopoietic exon translation (Ito et al. 1993). The testis and erythroid GATA-1 cDNA differ in the 5'UTR sequence only, suggesting that the expression of GATA-1 protein is identical in both tissues (testis and bone-marrow) (Ito et al. 1993). Therefore, to assess the role of GATA-1 protein in the testis, selective or partial inactivation becomes necessary (Viger *et al*. 2004). In this context, Yu and collaborators (2002) constructed a mouse model (ΔdblGATA), in which a targeted deletion of the palindromic GATA-1 site was performed, and GATA-1 gene deletion occurred in sequences from 691 to 671 bp upstream of the last

nucleotide in the first hematopoietic exon (IE). The alteration of this exon compromises the function of GATA-1 protein, helping to elucidate the functional role of GATA-1 in different tissues. The ΔdblGATA mouse strain has been widely used for studies of allergy and asthma. In the bone marrow of this mouse strain, it is known that deleting a high-affinity GATA-binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage (*in vivo*) and impairment of basophil development. Although this animal model is beneficial for immunological studies, the consequences of GATA-1 mutation for testis function remain to be elucidated.

Despite being crucial for hematopoiesis and cardiac function, very few studies have explored the role of GATA-1 in testicular homeostasis so far (Feng *et al*. 1998; Viger *et al*. 2004; Onodera *et al*. 1997; Ito *et al*. 1993; Yomogida *et al*. 1994; Labbaye *et al*. 2002; Buaas *et al*. 2004; Jiang and Wang 2004). Although GATA-1 expression has already been described in mice Sertoli cells and rat Leydig cells, its role in the testis is still not fully understood. In this context, this work aimed to study the involvement of GATA-1 protein in adult mice spermatogenesis in a more detailed way, investigating the testicular function of adult ΔdblGATA mice through detailed histomorphological, hormonal, genetic and *in vitro* analyses.

2. MATERIAL AND METHODS

2.1 ANIMALS

ΔdblGATA (BALB/c genetic background; Vieira *et al*. 2009) mice were obtained from the Immunopharmacology Laboratory at Federal University of Minas Gerais (UFMG), and BALB/c wild-type mice were obtained from Central Vivarium (CEBIO-UFMG, Brazil). All animals (n = 17 per group) were housed in a conventional specific pathogen-free facility at an average temperature of 22°C, 70% average humidity, and 12h photoperiod. Water and pelleted food were available *ad libitum*. Sexually mature animals were euthanized at 90 days of age. The current study was performed according to the Brazilian Government's ethical and animal experiments regulations (Law 11794/2008). All animal experiments were performed according to the Guidelines for Animal Use and Experimentation as set by the Animal Experimentation Ethics Committees from UFMG (CEUA/UFMG; #340/2019, Belo Horizonte, Brazil).

2.2 BrdU INJECTIONS

To estimate the duration of spermatogenesis, two animals from the ΔdblGATA group received intraperitoneal injections of BrdU (5-bromo2-deoxyuridine; 150mg/Kg BW) diluted in saline solution

using a hypodermic needle. BrdU is a specific marker of cells that are synthesizing DNA at the moment of injection. Each animal received two BrdU injections at 17 days and 1 hour before euthanasia.

2.3 TISSUE PREPARATION

All mice were euthanized by anesthetic overdose [ketamine (80 mg/Kg BW) and xylazine (10 mg/Kg BW); Sigma-Aldrich, St. Louis, MO, USA]. The testes, epididymis and seminal vesicles were collected and weighed. Since testicular density is very close to 1g/mL (França 1992), testicular volume was considered equal to its weight. The gonadosomatic index (GSI, testes total mass divided by body weight) was estimated for all investigated animals. The anogenital index (AGI) was measured using a digital caliper, and the values obtained were corrected for the cube root of the bodyweight (Auharek et al. 2012). Testes samples were fixed in solutions of 4% glutaraldehyde, Bouin's, or methacarn solutions. Six testes per group were frozen at -80°C for further use in qPCR analyses.

For morphometric analyses (n=6), the samples fixed in glutaraldehyde were dehydrated in ethanol and routinely embedded in glycol-methacrylate (Historesin, Leica). Histological sections, 4μm thick, were obtained using a glass knife with a rotatory microtome (Leica RM-2165) and stained with a toluidine blue solution in 1% sodium borate. For immunostaining (n=6), 6μm thick sections were obtained from samples fixed in Bouin's or methacarn solutions after dehydration in ethanol and embedding in paraplast® (Sigma-Aldrich).

2.4 HISTOMORPHOMETRIC ANALYSIS

2.4.1 Seminiferous tubule diameter and epithelium height

The mean diameter and epithelium height of the ST were measured in twenty round ST cross-sections, independent of the stage of the seminiferous epithelium cycle (SEC). All histomorphometric analyses were performed using Image J v.1.45s software (Image Processing and Analysis, in Java).

2.4.2 Volume densities of the testis parenchyma components

The testicular parenchyma components' volume densities (%) were calculated after counting 7,800 points over testis parenchyma. For this, we analyzed fifteen fields (images) obtained randomly from a continuous slide horizontal scanning using a graticule of 520 points. The intersections that coincided with tunica propria, seminiferous epithelium, tubular lumen, LCs, spermatogonia, connective tissue cells, blood, and lymphatic vessels were scored.

2.4.3 Germ and Sertoli cell numbers

To estimate the efficiency of spermatogenesis progression, we counted the cells present at stage VII of the spermatogenic cycle (Russell *et al*. 1990) in ten-round ST cross-sections for each animal at 100x magnification. These cell counts were corrected according to the method described by Abercrombie (1946) and modified by Amann (1962). Cell ratios/proportions were obtained from these corrected counts. Assuming no significant gem cell loss occurs during spermiogenesis, the number of round spermatids counted at Stage VII of the spermatogenic cycle was considered the final spermatozoa population (Russell and Clermont 1977; Hess and França 2007).

The total number of SCs per testis and testis gram were determined from the SC nucleoli number per tubule cross-section and the total length of ST, according to Hochereau-de Reviers and Lincoln (1978). Following this and considering the net weight of testis, the number of SCs per gram of testis was defined.

2.4.4 Stages of the seminiferous epithelium cy3cle

SEC stages were characterized based on the development of the acrosomic system and the morphology of the developing spermatid nucleus (Russell *et al*. 1990; Hess and França 2007). The relative stage frequencies were calculated by randomly evaluating 200 ST cross-sections per animal at 1000x magnification (Leal and França 2006).

2.4.5 Duration of spermatogenesis and daily sperm production

The duration of the spermatogenic cycle was estimated based on the stage frequencies and the most advanced germ cell type labeled in the two time periods following the BrdU injections (Lara *et al*. 2016). The daily sperm production (DSP) per testis and testis gram were achieved based on the following formula developed by França (1992): DSP = (total number of SCs per testis) x (the ratio of round spermatids per SCs at stage VII) x (stage VII relative frequency [%])/(stage VII duration [days]).

2.4.6 Leydig cell parameters

LC volume was obtained using the nuclear volume as well as the proportion between the nucleus and cytoplasm. For this purpose, 30 nuclei were measured per animal, and LC nucleus volume was obtained using the sphere formula ($4/3\pi R^3$, in which R = nuclear diameter/2). The proportion between the nucleus and cytoplasm was calculated after scoring 1000 points over LCs for each animal. The number of LCs was estimated from the LC size and the total volume occupied by these cells in the testis parenchyma (Costa *et al*. 2018).

2.5 IMMUNOSTAINING AND PIXEL INTENSITY QUANTIFICATION

The immunoperoxidase method was used for BrdU, CASPASE-3, 3βHSD1, GFRα-1, GATA-1 and 17βHSD3. For this purpose, serial sections were deparaffinized, rehydrated, and the antigens were exposed by heating in buffered sodium citrate (pH 6.0) at 96°C for 5 minutes (this step was dismissed for Caspase-3 staining). Subsequently, the endogenous peroxidase activity was blocked by incubating the sections in a 3% hydrogen peroxide solution (Sigma, St. Louis, MO, USA) for 10 min. Then, the sections were treated with Ultra V Block (Thermo Scientific, Fremont, CA, USA) for 5 minutes to block non-specific antibody binding sites. Samples were incubated overnight at 4°C with primary antibodies: anti-BrdU (1:200 dilution; sc-32323, Santa Cruz), anti-Caspase-3 (1:100 dilution; c8487, Sigma), anti-3βHSD1 (1:100 dilution; sc-30820, Santa Cruz), anti-GFRα-1 (1:50 dilution; sc-271546, Santa Cruz), anti-GATA-1 (1:200 dilution; ab-2818294, Thermofisher) and 17βHSD3 (1:50 dilution; ab126228), Abcam).

Reactions were visualized using biotin-conjugated secondary antibodies (for GFRA1 and 3βHSD1; 1:100 dilution, Abcam, ab6740, lot number GR27132-6), (for BrdU, 1:200 dilution, Imuny, IC1M02, lot number 16160), (for CASPASE-3, GATA-1 and 17βHSD3, 1:100 dilution, Abcam, ab93697, lot number GR313750-13) in combination with Elite ABC Kit (Vector Laboratories, CA, USA) and streptavidin (Thermo Scientific, TS-125-HR). Detection of the signal was performed by reaction with 3,3-diaminobenzidine (DAB, Sigma Aldrich). Negative controls had only the primary antibodies omitted. All the stained samples were analyzed using an Olympus microscope (BX60). Quantification of integrated pixel intensity of stained cells was calculated as the ratio between a stained region of interest inside a cell and the background, as previously described (Oliveira *et al*. 2020).

The immunofluorescence method was performed for CASPASE-3 (1:100 dilution; c8487, Sigma), GATA-1 (1:200 dilution, SC-362262, Santa Cruz) and GATA-4 (1:100 dilution; sc-25310, Santa Cruz) and visualized using a secondary antibody conjugated to Alexa Fluor 488 (1:400 dilution, IC-1M08; Thermo Fischer Scientific) in a Zeiss fluorescence microscope (ApoTome). The immunofluorescence was also performed in cultured Leydig cells (in coverslips). Briefly, the cells were fixed in 4% formaldehyde in PBS and treated with 1% of Sodium Dodecyl Sulfate (SDS) solution. The cells were incubated with a 1% BSA in PBS to block unspecific antibody binding. The primary antibody 17βHSD3 (1:50 dilution; ab62221, Abcam) was then added to the fixed cells (2 hours) at room temperature in a humidified chamber overnight at 4°C. After washing the dishes, the fluorescent conjugated secondary antibody (CruzFluor™ 488, 1:50 dilution; sc362262, SantaCruz) was supplemented at room temperature for 30-60 minutes. The DNA was stained with DAPI, and the

cells were fixed again using 4% formaldehyde. Finally, we added an anti-fading medium (ProLong®, Invitrogen) in the coverslip to mount the slides.

2.6 GENE EXPRESSION ANALYSIS

2.6.1 RNA extraction

Testicular tissue samples were kept in RNAlater Solution (Invitrogen) at -80°C until the moment of total RNA extraction. After maceration on dry ice, 1ml of Trizol® (Invitrogen) was added, quickly homogenizing the small testicular tissue fragments. Next, 200µL of chloroform (Sigma) was added to each sample and incubated for 3 minutes at room temperature. The homogenate was centrifuged at 12000g for 15 minutes at 4°C, and the colorless supernatant containing the RNA was carefully removed and transferred. After that, 500µL of isopropanol (Sigma) was added to the supernatant and incubated at 4°C for 20 minutes to obtain precipitated RNA. The RNA pellet was washed twice with chilled 75% ethanol (Sigma), centrifuged at 7500g for 2 minutes, and resuspended in DEPC water. RNA samples were treated with DNAse (DNA-free^{TM} DNA Removal kit, ThermoFisher Scientific) to avoid gDNA contamination. The integrity of the RNA was analyzed using agarose gel electrophoresis, and the RNA concentration in the samples was determined by fluorometry in Qbit® (Invitrogen).

2.6.2 cDNA synthesis and Real-Time PCR

2µL of total RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®). Primer sequences were standardized for concentration and annealing temperature via StepOnePlus Real-Time PCR System (Applied Biosystems®). All qPCR reactions were conducted using Maxima SYBR Green qPCR MasterMix with cDNA 1:10 and primers 600nM (Primer sequences can be found in Supplementary Table 1). The PCR protocol corresponds to 5 min at 95°C and 45 cycles with 1min at 60°C and 10s 95°C, each cycle. The fluorescence was detected at the end of each extension phase, and the melting curve was analyzed. The fold change of each sample was determined using *Gapdh* as an endogenous gene by the comparative method of ΔΔCt ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001). *Gapdh* was selected as the housekeeping gene based on the melting curves and analysis of its expression in different samples in which there was no Ct variation equal to or greater than 1.

2.7 CELL CULTURE

The cell culture was obtained according to the protocol described by Oh and colleagues (2017). Briefly, testes (n = 5 per group) were decapsulated in RPMI 1640 medium and digested in 0.5 mg/ml collagenase solution (type IV, Sigma-Aldrich) at 37 °C for 15 min. After, the supernatant containing

interstitial cells was collected and filtered (70 µm nylon cell strainer). Discontinuous Percoll density gradients (36% and 60% in PBS) were used to purify Leydig cells. Cells in the 36-60% interface were aspirated carefully and submitted to the *in vitro* culture using RPMI 1640 medium supplemented with 10% FBS for 24 hours. The cells were subsequently maintained in a serum-free medium for 12 hours to promote starvation before the human chorionic gonadotropin (hCG; 0.5 IU/ml; Sigma-Aldrich) stimulus. After one, two and three hours of the hCG stimulus, the supernatants were collected to estimate the testosterone levels.

2.8 HORMONAL ANALYSIS

2.8.1. Plasma testosterone levels

In order to perform the hormonal analysis, blood samples were collected via cardiac puncture when mice were still anesthetized before euthanasia. The plasma was separated by centrifugation at 2000rpm for 10 minutes and stored at -20ºC. Plasma testosterone concentration was measured by radioimmunoassay (RIA) using a commercial kit (Tecsa®, Belo Horizonte, MG, Brazil). Although RIA may cross-react with other steroids (Auchus, 2014), the samples were processed in the automated Cobas 8000 (Roche Diagnostics Inc., Indianapolis, IN, USA) platform for direct assessment of testosterone through electrochemiluminescence assay. The testosterone coefficients of variation (CV) intra- and inter-assay were, respectively, 1.1% and 1.5%.

2.8.2 LH levels

LH levels were measured by ultrasensitive ELISAs, adapted from previously described methods for LH assays (Aquino et al., 2017; Silva et al., 2020; Steyn, et al., 2013). The capture antibodies utilized were monoclonal anti-bovine LH-b subunit (518B7, University of California) at 1:2500. The detection antibodies utilized were rabbit anti-rLH (AFP240580Rb,NIDDK-NHPP) at 1:40000. The secondary antibody utilized was horseradish peroxidase-conjugated goat anti-rabbit IgG (P044801-2, Dako Pathology Solutions, Santa Clara, CA) at 1:2,000 in both assays.

A 96-well plate (9018,Corning, Kennebunk, ME) was covered with 50 mL of capture antibody diluted in PBS overnight at 4°C. The capture antibody was decanted, and wells were incubated with 200 mL of blocking buffer for 2 hours at room temperature (RT). A standard curve was generated via a twofold serial dilution of the reference preparation. The wells were incubated with 50 mL of standards or samples for 24 hours at RT. Afterwards, wells were incubated with 50 mL of detection antibody for 24 hours at 4°C. The wells were incubated with 50 mL of secondary antibody for 1.5 hours at RT. After that, wells were incubated with 100 mL of 2 mg/mL o-phenylenediamine dihydrochloride (P1526, Sigma-Aldrich) diluted in citrate–phosphate buffer (pH 5.0) containing

0.02% hydrogen peroxide for 45 minutes at RT. 50 mL of 3 M HCl was used to stop the reaction. The absorbance was determined at 490 nm with a microplate reader, and the wavelength of 650 nm was used for background correction. For LH ELISA, the lower limit of detection was 0.07 ng/mL, and the interassay and intrassay coefficients of variation were 10.4 and 3.3%, respectively (Aquino et al., 2017; Silva et al., 2020; Steyn, et al., 2013).

2.8.3. TESTOSTERONE LEVELS IN CELL CULTURE MEDIA

The collected medium was analyzed using the Kit Testosterone Test system - EIA protocol (Monobind inc., USA). For this, ten microliters of the calibrators, controls and samples were pipetted in each microwell of the plate. Following this, 50 microliters of working reagent (enzyme conjugate) were added. The plate was gently shaken for 30 seconds, and then 50 microliters of Biotin Testosterone reagent were supplemented and incubated for 60 minutes at room temperature. After this step, the contents of the entire plate were discarded and washed with 300 microliters of washing solution. One hundred microliters of the substrate working solution were added to all microwells and incubated for 15 minutes at room temperature. Finally, 50 microliters of the stop solution were added, and the plate was directed to the ELISA reader. The reading of each microcavity was taken in a wavelength of 450nm, using the reference wavelength of 620-630nm to minimize cavity imperfections. The absorbance of each duplicate represented the corresponding testosterone concentration (ng/mL).

2.9 SPERM ANALYSES

For the sperm analyses, epididymides were collected, weighed and manually dissected on a Petri dish containing commercial Dulbecco's modified eagle's medium (#12500-062; DMEM/F12 - Gibco, Grand Island, NY, USA). Samples were analyzed under a light microscope at 35.5°C (Vieira, 2019; Figueiredo et al., 2021). The sperm vitality and motility were analyzed as described by Vieira (2019). The sperm morphology was investigated through sperm smears stained with Hematoxylin-Eosin (HE), by assessing 200 spermatozoa regarding the head, midpiece and tail morphology. The sperm defects were identified, counted and classified (Vieira, 2019; Figueiredo et al., 2021).

2.10 STATISTICAL ANALYSIS

All data were tested for normality and homoscedasticity of the variances following Kolmogorov-Smirnov (Dallal-Wilkinson-Lilliefor) and Bartlett tests. Analyses were conducted using the graphics and statistics program PRISM v5.0 (GraphPad Software, Inc.). One-way ANOVA assessed data for comparisons within groups followed by Newman-Keuls test in case of normal distribution, or by

Kruskal-Wallis followed by Dunn's test in nonparametric data. Data were represented as the mean ± SEM (standard error of the mean), and differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1 Biometry and histomorphometric data

Biometric data collected from the investigated groups are depicted in Table 1. While both body and testis weights were significantly smaller in ΔdblGATA, the gonadosomatic index (Fig. 3G) was higher. Seminal vesicle weight and anogenital index were also smaller in ΔdblGATA. Morphometric parameters of testicular parenchyma are shown in Table 2. There were no significant differences regarding tubular diameter, seminiferous epithelium height, volume density of the tubular compartment components, and the length of the ST between the evaluated groups ($p > 0.05$). Concerning the intertubular compartment, the proportion of LCs and connective tissue was higher in ΔdblGATA mice, whereas blood vessels and lymphatic space proportions were smaller in this group ($p < 0.05$).

3.2 GATA-1 protein and expression in ΔdblGATA animal

Specific and robust immunolabeling for GATA-1 protein in Sertoli cells was observed through immunohistochemistry in control mice (Fig. 1A, 1E, 1E', 1E''). The testis of ΔdblGATA mouse also showed this protein expression in well-demarcated Sertoli cells, as shown in Figure 1B, 1F, 1F'and 1F''. Pixel analysis showed a difference in the expression (intensity) of GATA-1 proteins between the groups, being higher in the control when compared to the ΔdblGATA group (Fig. 1C). Labeled Sertoli cells (GATA1+) were seen in stages of the seminiferous epithelium cycle near spermiation. Regarding the Gata-1 gene relative expression, it was possible to observe a significant reduction (~75%) in ΔdblGATA animals (Fig. 1D).

3.3 Duration of spermatogenesis

The mean percentages of the stages of the SEC as well as the frequencies of the pre-meiotic (VII-XI), meiotic (Stage XII), and post-meiotic (Stages I-VI) phases are displayed in Fig. 1G. Statistical differences were observed concerning the frequencies of stages I, V, VI, VII, VIII, and XII ($p < 0.05$) (Fig. 1G).

The most advanced labeled germ cell types observed in the ΔdblGATA group in the different periods evaluated after BrdU injections are shown in Fig. 1H. Approximately one hour after the BrdU pulse, the most advanced germ cells labeled were pre-leptotene spermatocytes located in the basal

compartment at Stage VIII. Seventeen days after the BrdU injection, round spermatids were the most advanced germ cell labeled at stage VII/VIII.

Based on BrdU-labelings and stage frequencies (Fig. 1I), the mean duration of the SEC for ΔdblGATA mice was estimated as 8.67 ± 0.17 days. Since approximately 4.5 cycles are necessary for the spermatogenic process to be completed, the total duration of spermatogenesis in ΔdblGATA mice was estimated as 38.28 ± 0.76 days.

3.4 Sertoli and germ cell data

Concerning SCs, no differences were observed regarding their number per ST cross-sections at stage VII (Fig. 2A) or per testis gram (Fig. 2B) ($p > 0.05$). Additionally, the immunofluorescence for GATA-4 (Fig. 2C-D) showed a similar staining pattern of expression in the Sertoli cells in both investigated groups. Regarding germ cells, ΔdblGATA mice exhibited increased number and volume density of differentiated spermatogonia in the testicular parenchyma ($p < 0.05$; Fig. 2E-F). Aiming to investigate an undifferentiated spermatogonia molecular marker, we performed immunolabeling using the GFRA1 antibody. In the control group, single and paired GFRA1-positive spermatogonia were frequently observed in the ST cross-sections (2D dimension) (Fig. 2G). Differently, in ΔdblGATA animals, GFRA1-positive spermatogonia were primarily perceived as a single cell per ST cross-sections (Fig. 2H).

A higher incidence of spermatocyte apoptosis was noted in ΔdblGATA mice testis compared to the control, which was further confirmed by CASPASE-3 immunolabeling. Both immunoperoxidase (Fig. 2I-J) and immunofluorescence (Fig. 2K-L) assays revealed a high prevalence of Caspase-3-positive pre-leptotene spermatocytes in ΔdblGATA mice.

Considering the testicular cell ratios obtained (Fig. 2M-O), only the overall meiotic yield (number of round spermatids per differentiated spermatogonia) was statistically different, being lower in the ΔdblGATA mice ($p < 0.05$). Although it seems that SC efficiency (round spermatids per SC) and daily sperm production tended to be lower in ΔdblGATA, it was not statistically different in comparison to the control group (Fig. 2O-P).

3.5 Leydig cell data

ΔdblGATA mice exhibited a range of significant alterations in LC parameters (Fig. 3A-F). The transgenic mice presented smaller LC nuclear, cytoplasmatic, and, consequently, individual volumes ($p < 0.05$; Fig. 3C-E). However, the number of these cells per gram of testis was higher compared to the control group ($p < 0.05$; Fig. 3F). A reflex of potential changes in the steroidogenic pathway, lower anogenital index and seminal vesicle weight were observed in the transgenic group ($p < 0.05$, Fig. 3G-

H). To evaluate this question, we conducted a 3βHSD1 immunostaining, which showed that this enzyme, while observed in the control group, was not as evident in LCs from ΔdblGATA mice (Fig. 3I-J). Corroborating this observation, pixel intensity analysis of 3βHSD1 showed a reduced expression in ΔdblGATA mice (Fig. 3K). Similar immunostaining pattern was observed using anti-17βHSD3 (Fig. 3L-M). Since it is known that another protein of the GATA family (GATA-4) is involved in testosterone synthesis (Schrade *et al*. 2015), we performed immunolabeling to evaluate this protein in ΔdblGATA Leydig cells. Interestingly, GATA-4 protein in LC was noted in both groups (Fig. 3N-O), suggesting that the molecular downstream of GATA-1 may not be hampering the GATA-4 pathway.

Our genetic analyses (qPCR data) of steroidogenesis-related genes showed that *Ar, Star, Hsd3b1, Cyp17* and *Cyp19* presented a lower expression in ΔdblGATA mice (Fig. 3P). *Cyp11* and *Wt1* showed a similar relative expression between the control and transgenic groups. Only the *Insl3* gene showed a significant increase in the ΔdblGATA group. Confirming these findings, ΔdblGATA also presented elevated LH concentrations (Fig. 3Q) associated with reduced plasma testosterone levels (Fig. 3R).

Leydig cells were successfully cultivated and immunolabeled with 17βHSD3 (Fig. 4A-C). After extracting their RNA and evaluating the relative gene expression, it was possible to observe three specific genes, *Hsd3b1, Hsd3b6* and *Insl3*. There was a decrease in *Hsd3b1* (Fig. 4D) and *Hsd3b6* (Fig. 4 E) expressions, while *Insl3* showed a higher expression (Fig. 4F). The cell culture medium was collected, and testosterone levels were measured by ELISA assay. The result showed lower testosterone levels in the ΔdblGATA Leydig cell culture at all evaluated time points (Fig. 4G).

3.6 ΔdblGATA mice present similar values of sperm vitality and motility, but higher prevalence of spermatozoa with amorphous head

Sperm parameters are shown in Table 3. No statistically differences were observed regarding the sperm vitality and motility between the investigated groups. However, in comparison to controls, ΔdblGATA mice showed increased number of spermatozoa with amorphous head ($p < 0.05$) (Fig.4H-Q).

4. DISCUSSION

In the present study, through detailed histomorphological, hormonal, and genetic analyses, several testicular alterations resulting from the GATA-1 protein mutation were described for the first time in adult mice testis. It was also showed that even the ΔdblGATA animal has GATA-1 protein in Sertoli cells, as observed in control animals. However, this expression is weaker in mutant mice, suggesting that the mutation could decrease the production of this protein in the testis. Furthermore, because it is

a mutant protein with a lower expression, the GATA-1 function could be altered, generating some testicular changes, as demonstrated in the present study.

Previously, Yomogida and collaborators (1994) showed that GATA-1-positive Sertoli cells were found exclusively in cross-sections of seminiferous tubules at stages VII, VIII and IX, whereas seminiferous tubules in other stages were negative for GATA-1-staining in adult animals. We observed the same pattern, with only stages close to spermiation presented GATA1 positive Sertoli cells. Although daily sperm production was not altered, ΔdblGATA mice exhibited important tubular alterations. These mice have a significantly higher volume density of differentiated spermatogonia and an increased number of Caspase-3-positive pre-leptotene spermatocytes. However, the main significant changes occurred in the intertubular compartment, where LCs were profoundly affected by the presence of a dysfunctional GATA-1 protein. Differences were observed in their number, volume, and protein expression. Besides the lower level of plasmatic testosterone and the decreased testosterone level in the culture of Leydig cells, five genes related to the androgenic activity (*Star*, *Hsd3b1, Cyp17, Cyp19,* and *Ar*) presented lower expression, suggesting a crucial role of GATA-1 in the steroidogenic pathway in adult mice. The qPCR from the Leydig cell culture revealed a significant decrease in *Hsd3b1* and *Hsd3b6* expressions, which is consistent with the lower testosterone levels. According to Yokoyama et al. (2019), the *Hsd3b6* is a classical marker for adult Leydig cells. Confirming the genetic data, the 3βSHD1 showed weaker immunolabeling (pixel analysis) in the ΔdblGATA Leydig cells. The same immunostaining pattern was found for the 17βHSD3.

Only the *Insl3* gene expression was increased *in situ* and *in vitro*. Laki et al. (2019) demonstrated that the increase in *Insl3* gene expression is directly related to the hyperplasia of Leydig cells, a pattern observed in ΔdblGATA mice. Chen et al. (2014) showed that the crosstalk between Sertoli cells and Leydig cells has essential roles in Leydig cell steroidogenesis. Therefore, we can suggest that the steroidogenic pathway regulated by Wt1 continued preserved even with the mutation in the GATA-1 protein because no difference was observed for this gene expression. Further studies should focus on the crosstalk between LCs and hematopoietic cells in the testicular interstitium to investigate some signaling alterations.

GATA-1 expression is well described in SCs, and some *in vitro* studies have also demonstrated its expression in LCs (Zhang *et al*. 2002; Qamar *et al*. 2009). Indeed, our results suggest that GATA-1 is a critical factor for LCs physiology. ΔdblGATA mice exhibited impaired steroidogenesis, reflecting lower plasma testosterone levels, lower gonadosomatic index and seminal vesicle weight. Another morphofunctional feature that reflects the lower testosterone production is the reduced LC cytoplasmatic volume in ΔdblGATA mice, which can be associated with a reduced volume of smooth endoplasmic reticulum and lipid droplets accumulation (Johnson and Thompson 1987). The increased

LH levels in ΔdblGATA mice suggest that their Leydig cells are less functional than those from controls (indicating possible primary hypogonadism).

Concerning other transcription factors of the GATA family, it is already known that GATA-4 and GATA-6 play an essential role in the steroidogenic pathway (Viger *et al*. 2004; Schrade *et al*. 2015; Penny *et al*. 2017). Evaluating the presence of GATA-4 protein in ΔdblGATA mice, our transgenic model mice showed this transcription factor in LC nuclei. These findings suggest that GATA-1 mutation does not impair the GATA-4 activity. Thus, we may assume that even with the alteration of GATA-1, some steroidogenesis stimuli are maintained by the GATA-4-triggered pathway (George *et al*. 2015). Other GATA-family factors, such as GATA-2, have also been described as regulating steroidogenesis genes in the human placenta (Lai *et al*. 2017).

The lack of androgenic support through the ablation of androgen receptors arrests the development of the third phase of spermatogenesis, impairing spermatid differentiation (De Gendt *et al*. 2004). Interestingly, we observed a significant number of sperm head defects in the ΔdblGATA group. This data suggests that lower testosterone production in these animals may be affecting spermiogenesis. Although a significant reduction in plasmatic testosterone levels was detected in the present work, the spermatogenesis developed rather satisfactorily, and there was no significant difference in daily sperm production. This data suggests that intratesticular testosterone levels were sufficient to maintain the progression of spermatogenesis in ΔdblGATA mice. It is known that even low levels of intratesticular testosterone are still able to keep a healthy development of spermatogenesis (Cunningham and Huckins 1979). Future studies should be conducted investigating the intratesticular levels in ΔdblGATA mice.

Lindeboom and collaborators (2003) developed a specific conditional knockout of the GATA-1 gene in SCs. Surprisingly, their GATA-1-mutant testes were both morphologic and functionally normal, indicating that the absence of this factor, specifically in SCs, does not interrupt spermatogenesis. Herein, despite an imbalance observed in the spermatogonial phase, we observed a somehow similar feature. The spermatogenesis progressed without serious adverse effects in ΔdblGATA mice. Additionally, the number of SCs, SC efficiency and GATA-4 labeling were similar between the investigated groups. Conversely, the sperm morphology defects could indicate an altered SC function related to the spermatid's differentiation. No differences were observed concerning the duration of spermatogenesis (Oakberg 1956; Costa *et al*. 2018; Oliveira *et al*. 2020), indicating that GATA-1 does not influence the germ cell pace (França et al., 1998).

In ΔdblGATA mice, the increased number of differentiated spermatogonia suggests the involvement of GATA-1 specifically regulating the spermatogonial phase, whose signaling network is highly complex and deserves a cautionary discussion. Based on our cell counts and GFRA1 labeling pattern, it is suggested that altered GATA-1 promoted increased differentiation of the spermatogonial

stem cells (SSCs). It is also worth mentioning that GATA-1 interacts with PLZF in megakaryocyte development and erythroid cell production (Labbaye *et al*. 2002), meaning that there is a possibility that GATA-1 may be interacting with PLZF in the testis as well. Although we did not investigate this protein interaction, it is currently known that PLZF plays a crucial role in promoting SSCs self-renewal (Buaas *et al*. 2004). Furthermore, reduced androgen levels may be decreasing PLZF expression (Jiang and Wang 2014). In this context, one can say that ΔdblGATA mice present a predisposition towards SSCs differentiation. However, as the stock of SSCs is preserved, other routes are maintaining their self-renewal. Future studies are necessary to investigate, for instance, WNT5A signaling in promoting SSCs self-renewal because this factor is increased in situations associated with low testosterone levels (Tanaka *et al*. 2016).

Finally, the increased number of primary spermatocytes undergoing apoptosis may be related to a germ cell number control, possibly as a reflex of the extensive differentiation of SCCs leading to an excessive number of primary spermatocytes. It is known that SCs limit the number of germ cells that enter meiosis based on their support capacity (Lee *et al*. 1997; Murphy and Richburg 2014). Another exciting aspect is that apoptosis of primary spermatocytes occurred mainly at stages VII and VIII, when it is known that the expression of GATA-1 is accentuated in SCs (Ketola *et al*. 2002). Interestingly, these stages also presented significant differences regarding their frequency in the SEC in ΔdblGATA mice.

5. CONCLUSION

Several adverse effects on the testicular steroidogenic activity were demonstrated for the first time due to reduced functional GATA-1 protein. Reduced Leydig cell size, decreased expression of steroidogenic genes, smaller seminal vesicle, reduced anogenital index, diminished testosterone levels, and high LH levels were herein observed in adult ΔdblGATA mice. Concerning the tubular compartment, the main finding was the increased number of differentiated spermatogonia, which seems to be counterbalanced by increased primary spermatocyte apoptosis. The sperm analyses indicated a high frequency of sperm head defects, suggesting alterations in spermiogenesis (phase highly dependent on androgens). In sum, our results show the critical role that GATA-1 plays in steroidogenesis and testis function.

6. Compliance with ethical standards

6.1 Conflict of interest

No conflict of interest was declared.

6.2 Funding

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6.3 Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals followed the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee on Animal Use from the Federal University of Minas Gerais - CEUA document #340/2019).

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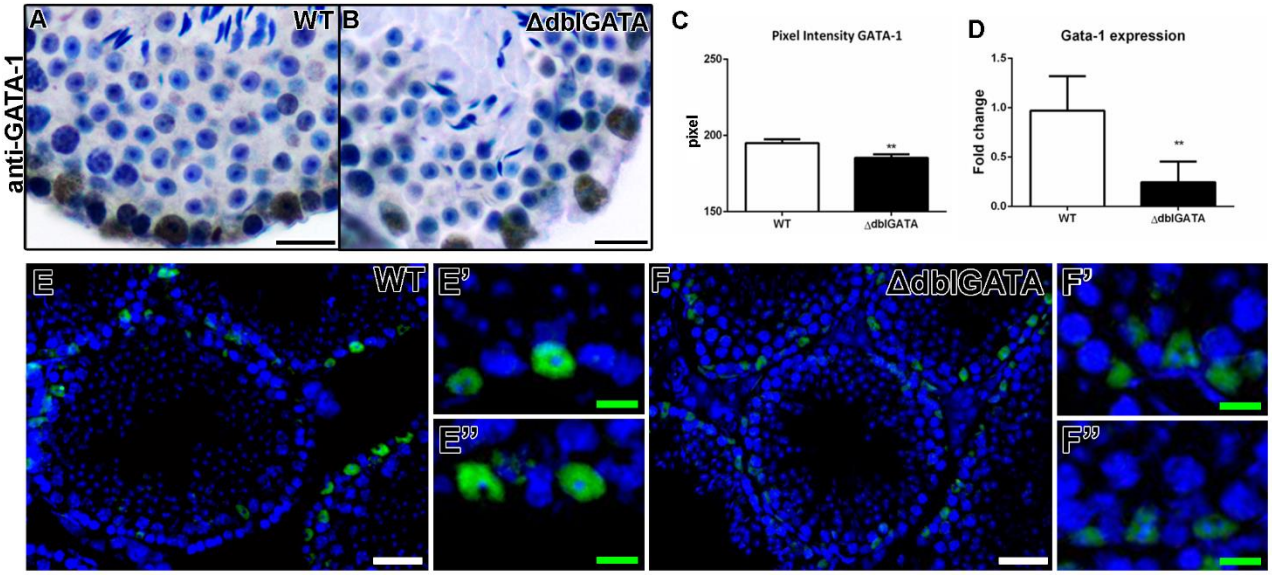
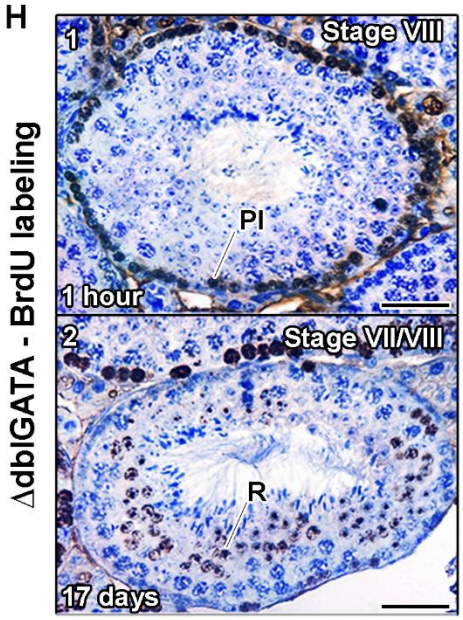
8. FIGURE LEGENDS



G

Stages of the SEC	WT	ΔdblGATA	
I	10.1 ± 0.8	3.5 ± 1.1	*
II-III	10.1 ± 0.7	13.85 ± 1.8	
IV	4.2 ± 0.3	4.99 ± 0.9	
V	8.1 ± 0.7	3.98 ± 0.4	*
VI	14.2 ± 1.2	8.62 ± 1.1	*
VII	19.5 ± 0.8	12.05 ± 1.6	*
VIII	5.4 ± 0.4	15.40 ± 2.1	*
IX	10 ± 0.7	13.05 ± 1.4	
X	7.7 ± 0.5	7.31 ± 1.4	
XI	8.2 ± 0.7	6.86 ± 0.9	
XII	2.3 ± 0.3	10.14 ± 1.9	*
Pre-meiotic (VII-XI)	50.9 ± 0.8	54.67 ± 2.1	
Pós-meiotic (I-VI)	46.7 ± 1.2	35.19 ± 1.8	
Meiotic (XII)	2.3 ± 0.3	10.14 ± 1.9	



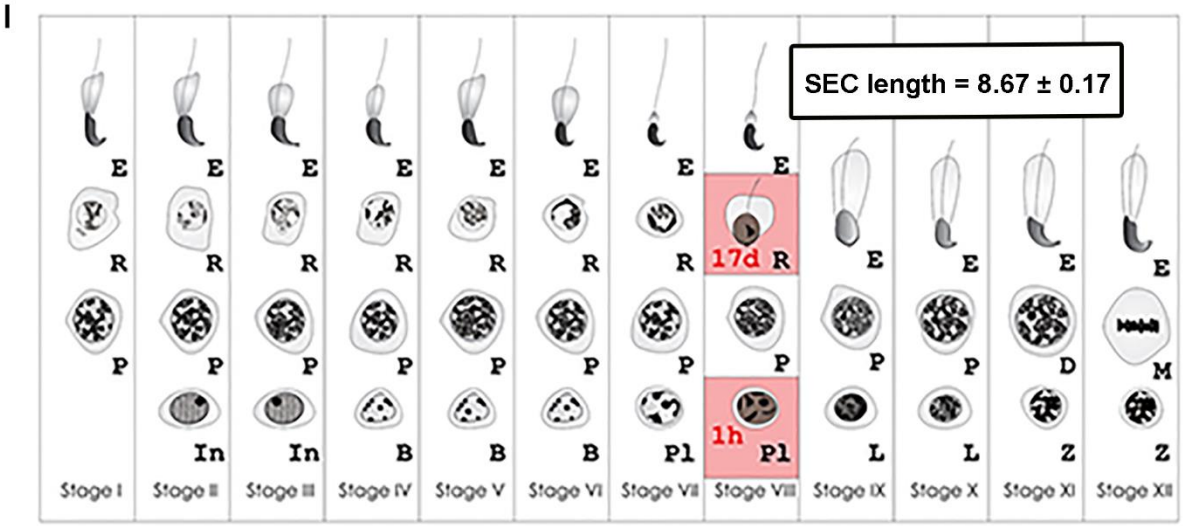


Figure 1 - GATA-1 expression and duration of the seminiferous epithelium cycle in ΔdblGATA mice. Immunolabeling for GATA-1 protein in Sertoli cells through immunoperoxidase (A-B). The intensity (in pixels) of GATA-1 proteins is lower in ΔdblGATA compared to control mice (C). Gata-1 expression in control and ΔdblGATA mice testes (D). GATA-1 protein immunofluorescence in Sertoli cells from controls (E-E'') and ΔdblGATA mice (F-F''). Relative frequency of stages of the seminiferous epithelium cycle (G). Immunoperoxidase (H) and schematic diagram (I) of labeled germ cells one hour (H1) and 17 days (H2) after BrdU injections. In = intermediate spermatogonia; B = type B spermatogonia; Pl = pre-leptotene; L = leptotene; Z = zygote; P = pachytene; D = diplotene; M = Meiosis; R: round spermatids; E = elongated sperm; SEC = seminiferous epithelium cycle. White and black scale bars = 50 µm. Green bars = 10 µm.

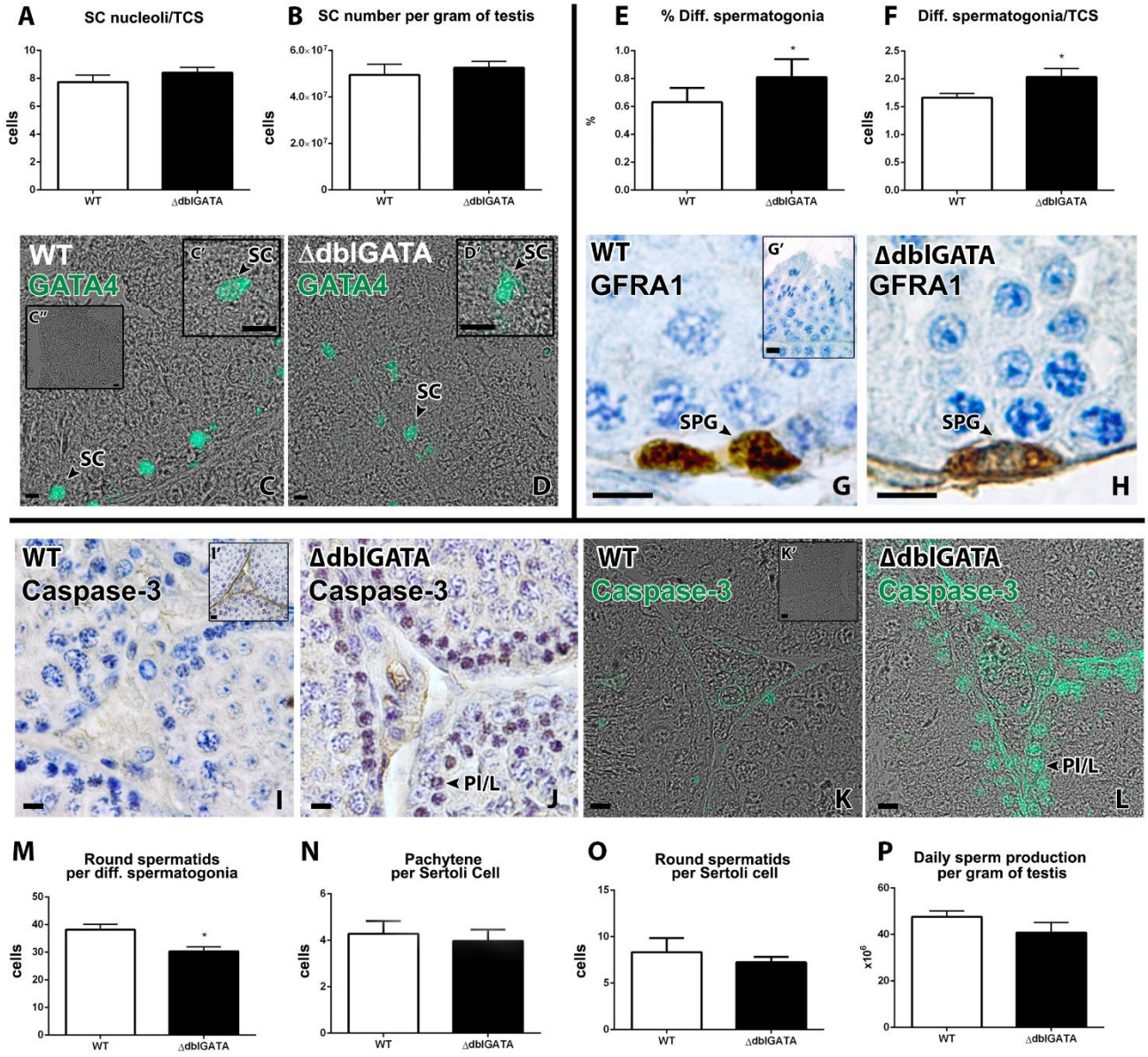
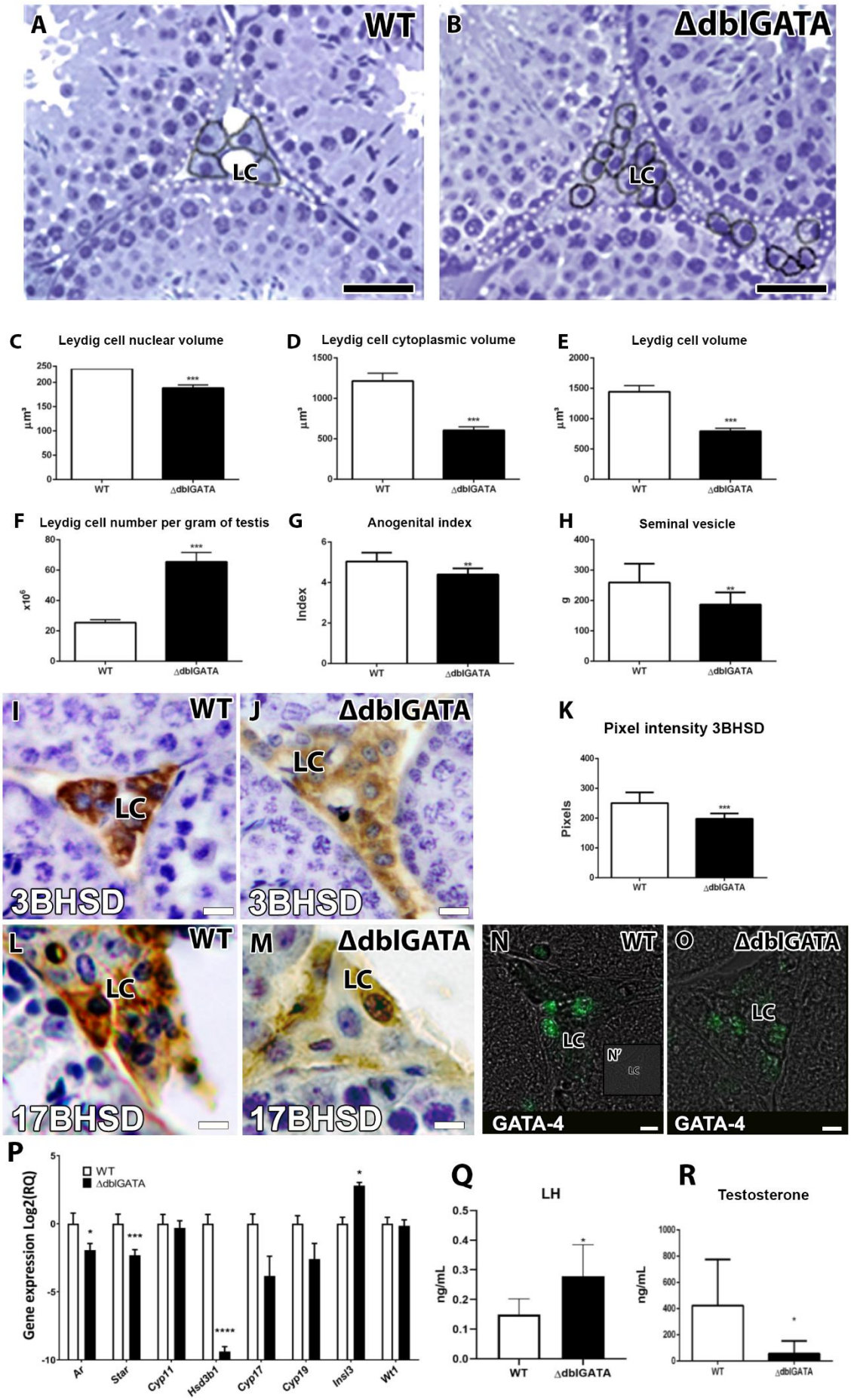


Figure 2 - Analysis of the tubular compartment in wild and ΔdblGATA mice. The number of Sertoli cells per cross-section of seminiferous tubules at stage VII of the seminiferous epithelium cycle (A). The total number of Sertoli cells per gram of testis (B). Immunofluorescences for GATA-4 in WT (C-D) and ΔdblGATA (E-F) mice. Immunoperoxidase for GFRA1 in WT (G-H) and ΔdblGATA (K-L) mice. Immunoperoxidase and immunofluorescence for CASPASE3 in WT (M and O) and ΔdblGATA (N and P) mice. Cellular ratios of different germ cell types per Sertoli cell (Q-S). Daily sperm production per gram of testis (T). Scale bars = 10 µm.


A
WT
LC
B
ΔdblGATA
LC
C
Leydig cell nuclear volume
µm³
WT
ΔdblGATA
D
Leydig cell cytoplasmic volume
µm³
WT
ΔdblGATA
E
Leydig cell volume
µm³
WT
ΔdblGATA
F
Leydig cell number per gram of testis
x10⁶
WT
ΔdblGATA
G
Anogenital index
Index
WT
ΔdblGATA
H
Seminal vesicle
g
WT
ΔdblGATA
I
WT
LC
3BHSD
J
ΔdblGATA
LC
3BHSD
K
Pixel intensity 3BHSD
Pixels
WT
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L
WT
LC
17BHSD
M
ΔdblGATA
LC
17BHSD
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GATA-4
O
ΔdblGATA
LC
GATA-4
P
WT
ΔdblGATA
Gene expression Log2(RQ)
Ar
Star
Cyp11
Hsd3b1
Cyp17
Cyp19
Insl3
Wt1
Q
LH
ng/mL
WT
ΔdblGATA
R
Testosterone
ng/mL
WT
ΔdblGATA

Figure 3 - Analysis of the intertubular compartment in wild-type and ΔdblGATA mice. Testicular histology showing the difference in Leydig cell volume between groups (A-B). Nuclear, cytoplasmic and total Leydig cell volume (C-E). The total number of Leydig cells per gram of testis (F). Anogenital index and seminal vesicle weight in both groups (G - H). Immunoperoxidase for 3βHSD1 in wild type (I) and ΔdblGATA mice (J). The pixel intensity of 3βHSD1 staining (K). Immunoperoxidase for 17βHSD3 in wild type (L) and ΔdblGATA mice (M). Immunofluorescence for GATA-4 in Leydig cells (N-O). qPCR technique, showing the relative expression of the following genes: *Ar, Star, Cyp11, Hsd3b1, Cyp17, Cyp19, Insl3 and Wt1* in the studied groups (P). LH (Q) and Serum testosterone (R) levels. Black scale bars = 50 µm. White scale bars = 10 µm.

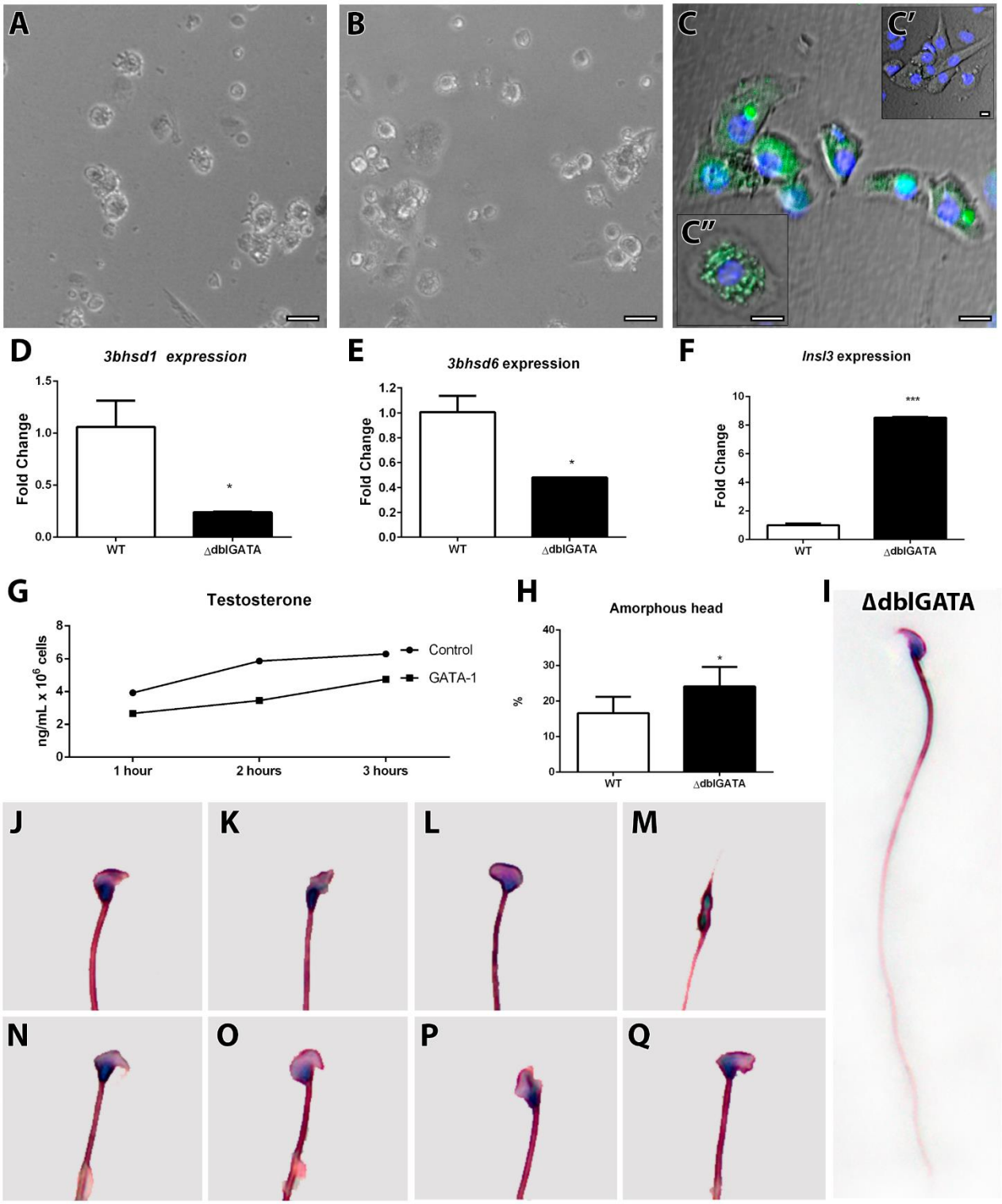

Figure 4 – Wild-type and ΔdblGATA mice Leydig cell culture and sperm morphology. Leydig cell culture images from wild-type and ΔdblGATA mice (A-B). 17BSHD3 immunofluorescence confirming the presence of Leydig cells in the culture system (C). Negative control (C'). Insert highlighting the immunolabeling in Leydig cell cytoplasm (C"). Altered gene expression in isolated Leydig cells (D-F). Levels of testosterone in the RPMI media in WT and ΔdblGATA Leydig cell

culture (G). Frequency of amorphous head in WT and ΔdblGATA mice (H). Normal sperm morphology in ΔdblGATA mice (I). Sperm head defects in ΔdblGATA mice (J-Q). White scale bars = 10 µm. Black scale bars = 5 µm.

9. Tables

Table 1. Biometric data from control and ΔdblGATA mice (mean ± SEM).

Parameter	**Control (n = 12)**	**ΔdblGATA (n=12)**
Body weight (g)	28.4 ± 0.7	22.9 ± 0.5*
Testis weight (mg)	104.1 ± 1.5	89.2 ± 1.2*
Gonadosomatic Index (%)	0.74 ± 0.02	0.78 ± 0.01*
Epididymis weight (mg)	35.9 ± 2.8	32.3 ± 1.8
Seminal Vesicle weight (mg)	259 ± 19	187 ± 11*
Anogenital Index (mm)	5.0 ± 0.11	4.4 ± 0.13*

n = number of mice. * Statistically significant differences ($p < 0.05$).

Table 2. Histomorphometric testicular data from control and ΔdblGATA mice (mean ± SEM).

Parameter	**Control (n = 6)**	**ΔdblGATA (n = 6)**
Tubular diameter (µm)	220 ± 2.6	218 ± 3.6
Seminiferous epithelium height (µm)	76.9 ± 1.1	74.3 ± 1.2
Volume Density (%)		
Tubular Compartment	93.1 ± 0.5	93.1 ± 0.4
Tunica propria	3.6 ± 0.2	3.7 ± 0.3
Seminiferous epithelium	80.4 ± 0.7	80.4 ± 0.9

Lumen	9.1 ± 0.7	9.0 ± 0.9
Intertubular Compartment	6.9 ± 0.4	6.9 ± 0.4
Leydig Cell	4.2 ± 0.4	5.1 ± 0.3*
Blood Vessels	2.0 ± 0.3	0.9 ± 0.09*
Lymphatic Space	0.6 ± 0.1	0.3 ± 0.1*
Connective Tissue	0.3 ± 0.05	0.6 ± 0.1*
Tubular length/testis (m)	2.4 ± 0.1	2.2 ± 0.1
Tubular length/g/testis (m)	24.6 ± 0.6	25 ± 0.8

n = number of mice. *Statistically significant differences ($p < 0.05$).

Table 3. Sperm parameters from control and ΔdblGATA mice (mean ± SEM).

Sperm Parameters	**Control (n = 6)**	**ΔdblGATA (n = 6)**
Sperm vitality (%)	29.3 ± 3.5	29.7 ± 4.0
Dead sperm	70.7 ± 3.5	70.3 ± 4.0
Sperm motility (%)	27.6 ± 4.5	30.9 ± 2.7
Progressive	8.2 ± 1.3	7.9 ± 2.0
Non-progressive	19.4 ± 3.8	23.0 ± 2.0
Immobile	72.4 ± 4.5	69.1 ± 2.7
Normal sperm (%)	20.3 ± 4.8	16.9 ± 7.0
Sperm defects (%)	79.7 ± 4.8	83.1 ± 7.0
Head defects	44.5 ± 3.5	49.9 ± 5.6
Amorphous head	16.6 ± 4.6	24.1 ± 5.5*
Midpiece defects	14.8 ± 3.5	15.7 ± 2.7
Tail defects	15.9 ± 0.9	12.9 ± 1.3

Without midpiece and tail	4.6 ± 1.6	4.7 ± 2.1

n = number of mice. *Statistically significant differences ($p < 0.05$).

Supplementary Table 1. Primer sequences of the present study.

Genes	**Foward**	**Reverse**
Ar	5’-CTGGGAAGGGTCTACCCAC-3’	5’-GGTGCTATGTTAGCGGCCTC-3’
Star	5’ATGTTCCTCGCTACGTTCAAG 3’	5’ CCCAGTGCTCTCCAGTTGAG 3’
Hsd3b1	5’ TGGACAAAGTATTCCGACCAG 3’	5’ GGCACACTTGCTTGAACACAG 3’
Cyp11	5’ AGGTCCTTCAATGAGATCCCTT3’	5’ TCCCTGTAAATGGGGCCATAC 3’
Cyp17	5’ GCCCAAGTCAAAGACACCTAAT 3’	5’ GTACCCAGGCGAAGAGAATAGA 3’
Cyp19	5’ ATGTTCTTGGAAATGCTGAACCC 3’	5’ AGGACCTGGTATTGAAGACGAG 3’
Gapdh	5’-AGGTCGGTGTGAACGGATTTG-3’	5’-TGTAGACCATGTAGTTGAGGTCA-3’

Artigo 2

Testicular function after post-transcriptional silencing of Sry gene

Artigo 2 - Testicular function after post-transcriptional silencing of Sry gene

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Abstract

The Sry gene is located in the Y sexual chromosome, and its expression is crucial for male sex determination at the mammalian embryonic stage. Besides its importance in embryos, Sry transcripts can be found in several tissues in adult animals, including testis. Although several hypotheses have been raised about the role of the Sry gene in adult animals' testicular physiology, only a few pieces of evidence have been conclusive. Therefore, this project aimed to analyze the morphological and functional aspects of adult male mice treated with siRNA, promoting the silencing of Sry RNAm. siRNA was associated with carbon nanotubes and injected into the tail vein of Balb-C mice. The testis, epididymis and seminal vesicles were collected three days after the injection of the nanocomplex, fixed, and then embedded in paraplast and methacrylate. To elucidate the specific role of Sry gene expression in spermatogenesis, we performed Laser Doppler analyses, testicular morphometry, immunohistochemistry, TUNEL, and qPCR assays. The silencing of the Sry gene resulted in testicular blood flow alteration, the release of germ cells from the seminiferous epithelium, Sertoli and germ cell apoptosis, thickening of tunica propria, and Leydig cells hypertrophy. Further investigations on the Renin-Angiotensin System and the dysregulation of temperature are critical for this study. However, we demonstrated for the first time that the Sry gene is essential for spermatogenesis homeostasis in adult animals.

Keywords: *Sry*, siRNA, testicles, spermatogenesis, mice.

1. Introduction

Sry is a gene located on the Y chromosome, and its expression is necessary for the differentiation of the bipotential genital ridges in mammalian testis. The testis development in XX mice with a single addition of Sry in embryos confirmed this concept (Koopman et al., 1991). SRY initiates testis development by binding to a specific activating segment of Sox9 (Sekido & Lovell-Badge, 2008), a gene with a highly conserved role for testicular development. Surprisingly, for a gene with such profound relevance for testis development, Sry expression is only needed in a small population of the genital crest cells, for a short period, to start the development of the male gonad (Albrecht & Eicher, 2001). In mice, Sry gene expression occurs 10.5 days post-coitus (dpc) and is restricted to the central region of the genital crest. Subsequently, with 11.5 dpc, it expands to fill the entire gonad before becoming undetectable with 12.5 dpc (Bullejos & Koopman, 2001; Jeske et al., 1996). This small expression window is critical, and a delay of just a few hours can lead to an ovary or ovotestis development (Hiramatsu et al., 2009). Studies have shown that the levels of Sry expression need to reach a minimum limit to induce the testis development (Nagamine et al., 1999). Poor Sry expression results in abnormal testis development in XY embryos (Tevosian et al., 2002). Since regulation of Sry is also essential in humans, a better understanding of factors and pathways that regulate Sry may explain disorders related to sexual development and infertilities with undefined etiologies (Larney et al., 2015).

Studies of the Sry gene show that besides its participation in sexual determination, it is expressed in the testis of adult mammals, such as mice, humans and marsupials (Sinclair et al., 1990; Foster et al., 1992). Although poor explored, it is suggested that the product of this gene may be required for testicular physiology. In mice, for example, Sry is expressed by somatic cells in the genital crest and by the germ cells in adult animal testes (Hacker et al., 1995). The genes found on the Y chromosome are mainly expressed during spermatogenesis, and it is still unknown whether Sry expression could reflect the general transcription of this chromosome (Capel et al. 1993). It is known that Sry has two transcripts (mRNA), a circular mRNA that probably would not be efficiently translated, and a linear mRNA (derived from a single exon) that would have a more accessible translation. The linear Sry transcript on the genital crest is different from the circular transcript found in the adult testis (Hacker et al. 1995). Therefore, further studies are needed to identify the role of the Sry gene transcripts in the testis of adult animals. Recent data suggest that the Sry expression would be fundamental to modulating the body's vasomotion, reflecting in the animal's fertility.

Once activated in the genital ridges, Sry can trigger specific genes to initiate testicular differentiation (Hacker et al., 1995; Jeske et al., 1995). Besides Sox9 (Vidal et al., 2001; Bishop et al., 2000; Qin & Bishop, 2005; Sekido R. et al., 2004; She & Yang, 2016), additional genes, including

Wt1 (Englert, 1998; Hammes et al., 2001; Ito et al., 2006; Toyooka et al., 1998) and Sf1 (Pilon et al., 2003) were identified as part of this complex process. Another gene that effectively participates in testicular differentiation is Dmrt1 (Huang et al., 2017). It is known that the depletion of this gene in adult mice results in the appearance of the FOXL2 protein (responsible for ovarian differentiation) that leads to the transformation of the testis into an ovary (Barrionuevo et al., 2016). Likewise, the deletion of the Foxl2 gene in females promotes the transdifferentiation of granulosa cells into Sertoli cells (Huang et al., 2017). Foxl2 and Dmrt1 act as a Yin and Yang in maintaining the sexual identity of the gonads. It is important to observe that the ability of the gonads to transdifferentiate itself, modulating only one gene (even in an adult animal), has significant implications for basic and applied science (Huang et al., 2017).

In the present work, carbon nanotubes were associated with specific siRNA against the mRNA from the Sry gene to evaluate the consequences of this post-translational inhibition. This procedure is crucial because the production of Sry knockout animals would lead to offspring containing only females. The genomic silencing by siRNA is considered post-transcriptional, as it does not prevent the target gene from being transcribed. The interfering RNA can bind to the aimed mRNA, preventing it from being translated or even promoting its degradation (Napoli et al., 1990; Mello & Conte, 2004; Wu et al., 2012). Carbon nanotubes were chosen as vehicles due to their needle shape and the ability to cross the cellular plasmatic membrane, delivering the siRNA inside cells (Varkouhi et al., 2011). Therefore, we will assess this gene's importance in the adult animal's testicular physiology through this inhibition of the Sry mRNA translation.

2. Material and Methods

2.1 Animals

For the experiments, 20 wild male mice of Balb-c strain (*wild type)*, aged approximately 90 days (sexually mature animals), from the vivarium of the Institute of Biological Sciences of UFMG, were used. These animals were divided into three groups, being two control groups: wild animals (without injection) (n = 6) (Fig1; A) and injected with carbon nanotubes (n = 6) (Fig1; B). The treated group (Fig1; C) received carbon nanotubes associated with siRNA (n = 8). The experimental procedures were approved by the Ethics Committee on the Use of Animals (Protocol CEUA: 279/2016) – UFMG.

2.2 Preparation and siRNA administration

Specific sites within the mouse Sry gene were selected and evaluated on the NCBI Blast to confirm primers' specificity for siRNA studies. According to the manufacturer's instructions, the siRNA for Sry was synthesized by a method based on transcription using the Silencer kit (Ambion Inc., Austin, TX). The sequence of siRNA's forward and reverse oligonucleotides are, respectively: 5'AAGTGCCACAGAGGAGTTATACCTGTCTC3' and 5'AATATAACTCCTCTGTGGCACCCTGTCTC3'.

Subsequently, the synthesized siRNA was associated with carbon nanotubes (ntc), which functioned as a vehicle to transport and deliver the siRNA to the animal's cells. Due to its low water solubility, the carbon nanotube (single wall) was functionalized with a hydroxyl group (Ladeira et al., 2010) to increase its solubility and decrease its tendency to aggregate. Additionally, they were kept in an ultrasonic water bath (Elma Elmasonic) for 3 hours to disperse the carbon nanotubes particles. Afterward, the nanotubes (100 μg / mL) were combined with the siRNA molecules (100 nM) and again placed in the ultrasonic bath for 30 minutes to maximize this connection. Finally, the ntc + siRNA complex was injected into the tail vein of the mice.

2.3 Collection and preparation of the biological material

Considering that the siRNA half-life varies from 48 to 72 hours, the animals were euthanized through an anesthetic overdose (Ketamine (80 mg/Kg BW) + Xylazine (10 mg/Kg BW) three days after the application of ntc + siRNA complex (Fig 1A). As controls, we used animals with no injection and animals which received only carbon nanotubes. Afterward, the testis, epididymis, and seminal vesicle were collected and weighed. The mice testes were fixed by immersion in three different fixatives: glutaraldehyde (4% in 0.05M cacodylate buffer, pH 7.3), bouin, and methacarn. Additionally, some testes were frozen at -80° C for posterior use in qPCR techniques.

Testicular fragments, fixed in glutaraldehyde (stored for 12 hours at 4° C), were directed for light microscopy studies. Testes were dehydrated in increasing concentrations of alcohol (70º, 80º, 90º and 100º). After the dehydration, the fragments were embedded in plastic resin glycol-methacrylate based (Leica Historesin Embedding Kit, Leica Instruments) and subsequently sectioned at a thickness of 4 μm in a microtome (Leica RM-2165) with glass knives. The obtained sections were stained with 1% toluidine-borate sodium blue, mounted with Entellan (Merck), and analyzed under an Olympus BX41 and BX60 microscope.

The testicular fragments fixed in bouin and methacarn solution were directed for immunohistochemistry assays. The material was dehydrated, embedded in Paraplast(R), and sectioned at 6 µm of thickness. These sections have adhered to slides using poly-L-lysine. The photographic documentation was made using an Olympus BX60 photomicroscope with U-PlanApochromatic lenses and Olympus UC50 digital video camera system. The image analysis was done using the software ImageJ (NIH).

2.4 Testis morphometric analysis

2.4.1 Volumetric density

The volumetric density was estimated using the ImageJ software (NIH) using 520 intersections (points) per field in a 200x magnification. For each animal, 15 fields were randomly chosen, comprising a total of 7800 points. From the tubular compartment, tunica propria, seminiferous epithelium, apoptotic germ cells were evaluated. In the intertubular compartment, the analyses were focused on Leydig cells, connective tissue, blood vessels, and lymphatic space. The volume of each testicular component, expressed in mL, was estimated based on knowledge of the liquid volume of the testis. As the testis density is close to 1 (1.03 to 1.04; França, 1991), the weight of the testis was considered equal to its volume.

2.4.2 Tubular diameter and seminiferous epithelium height

The average tubular diameter per animal was obtained from the random measurement of 20 cross-sections of seminiferous tubules, using a calibrated ruler with 200x magnification. In the same sections, the seminiferous epithelium's height was assessed.

2.4.3 Nuclear volume and number of Sertoli cell

The nuclear diameters of Sertoli cells were estimated using the ImageJ software (NIH) using a calibrated ruler for 200x magnification. Two perpendicular lines inside the Sertoli cell nucleus were measured, and their average value was calculated in µm (Auharek & França, 2010). Thirty nuclei of Sertoli cells were assessed for each animal; their volume (µm³) was estimated based on the volume formula: Volume = 4/3πR3. The radius (R) corresponds to half of the nuclear diameter. The number

of Sertoli cells per testis was estimated from the testis weight, percentage of the nuclei obtained in volumetric density, and the nuclear volume of Sertoli cells (Costa et al., 2018).

2.4.4 Diameter and nuclear volume of the Leydig cell

The Leydig cell's nuclear diameters were estimated similarly to Sertoli cells, using the ImageJ software using a calibrated ruler for 200x magnification. Thirty Leydig cell nuclei were measured for each animal. The total nuclear volume (μm³) was estimated from the nuclear diameter value. This measurement was divided in half to acquire the radius value. Then this number was inserted in the formula presented in item 2.4.3.

2.4.5 Individual volume and number of Leydig cells

The volumetric density of the cytoplasm and the nucleus of the Leydig cell were estimated using the ImageJ software (NIH) using a grid with 1000 intersections (points) in 200x magnification. From this data, the volume (μm³) of each Leydig cell per animal was calculated, according to Costa et al. (2018). Aware of the individual and total volume of Leydig cells per testis, the number of these cells per animal was calculated.

2.5 Immunohistochemistry

The immunoperoxidase method was used for SRY, SOX9, CASPASE-3, and CONNEXIN-43. For this purpose, serial sections were deparaffinized, rehydrated, and the antigens were exposed by heating in buffered sodium citrate (pH 6.0) at 96°C for 5 minutes (this step was dismissed for Caspase-3 staining). Subsequently, the endogenous peroxidase activity was blocked by incubating the sections in a 3% hydrogen peroxide solution (Sigma, St. Louis, MO, USA) for 10 min. Then, the sections were treated with Ultra V Block (Thermo Scientific, Fremont, CA, USA) for 5 minutes to block non-specific antibody binding sites. Samples were incubated overnight at 4°C with the following primary antibodies:

- anti-SRY antibody (goat, 1:25, sc-8233, Santa Cruz Biotechnology);
- anti-SOX9 antibody (rabbit, 1:50, sc-20095, Santa Cruz Biotechnology);
- anti-CASPASE-3 (rabbit, 1:100; c8487, Sigma);
- and anti-CONNEXIN-43 antibody (rabbit, 1:100, sc-9051, Santa Cruz).

After the incubation, reactions were visualized using the following biotin-conjugated secondary antibodies:

- anti-goat for SRY (1:100, ab6740, Abcam)
- anti-rabbit for SOX9, CASPASE-3, and CONNEXIN-43 (1:100, ab93697, Abcam).

To detect the signals, we used the Elite ABC Kit (Vector Laboratories, CA, USA) and streptavidin (Thermo Scientific, TS-125-HR). We performed the reaction using 3,3-diaminobenzidine (DAB, Sigma Aldrich), and negative controls had the primary antibodies omitted. All the stained samples were analyzed using an Olympus microscope (BX60).

2.6 Genetic Analysis

2.6.1 RNA Extraction

The testicular tissue samples were kept in RNAlaterTM Solution (Invitrogen) at -80 °C until total RNA extraction. Therefore, after pounding the tissue on dry ice, 1 ml of Trizol® (Invitrogen) was added, quickly homogenizing the small fragments. Then, 200µL of chloroform (Sigma) was added to each sample. Microtubes were vigorously shaken and incubated for 3 minutes at room temperature. The homogenate was centrifuged (12000 g) for 15 minutes at 4 ° C, and the transparent supernatant containing the mRNA was carefully removed and transferred to a new microtube.

In the next step, 500 µL of isopropanol (Sigma) was added to the supernatant, incubated at 4 ° C for 20 minutes to cause mRNA precipitation. After another centrifugation (12000 g, 15 minutes, at 4°C), the supernatant was discarded, and the mRNA pellet was washed twice with 1 ml of 75% cooled ethanol (Sigma), vortexed and centrifuged at 7500 g for 2 minutes in each wash.

Finally, after drying for 1 hour, the mRNA pellet was resuspended in water treated with diethylpyrocarbonate (DEPC). The integrity of the RNA obtained was analyzed using agarose gel electrophoresis, and the concentration of the RNA samples was determined by fluorometry in Qbit® (Invitrogen).

2.6.2 RT-qPCR

From the total RNA extracted from testis of treated and control animals, cDNA was obtained through reverse transcription followed by polymerase chain reaction using M-MuLV enzyme and Random primers (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem®). To measure

gene expression using q-PCR, pairs of primers were designed for different sequences of interest (eg Sry, Sox9, Dmrt1, Caspase3, Ar, Star, Claudin11, Vegf, Gapdh and Beta-actin) using the "Real Time PCR" tool from Integrated DNA Technologies (Table 1).

The primers were then standardized on the StepOnePlus Real-Time PCR System (Life) at different concentrations and annealing temperatures. All quantitative PCR reactions were conducted on a 7500 Fast RealTime PCR System (Applied Biosystems) equipment using the SYBR Green® method.

2.7 Laser Doppler flowmetry (LDF)

Perfusion images acquired by Doppler laser (Moor Instruments, Ltd., England) were used to estimate testis' blood flow. The Doppler laser perfusion imaging equipment is used for non-invasive (and non-physical) visualization of blood circulation from the emission of a monochromatic laser, which is dispersed by the moving red blood cells. The technique allows the observation of changes in flow over time or assess differences in the flow of a specific area. The blood flow was estimated from changes in the frequency of the laser that is correspondent to red blood cells speed.

After scanning the testis, the tissue perfusion was coded on a color map. Minor or perfusion absence is represented by the dark blue color and the maximum perfusion by the red color. After digitization, the images were analyzed using the Moor LDI Laser Doppler 5.3 software to quantify blood flow. The results were obtained from the resolution ratio divided by the scrotum area of each animal. Due to the necessity to remain steady during the process, the animals were anesthetized with ketamine solution (60 mg / kg) and xylazine (5 mg / kg). The animals were then placed on a heating bed to maintain a constant temperature for 5 minutes before being subjected to the laser.

2.8 TUNEL

Through the TUNEL assay (Terminal dUTP Nick-End Labeling), the DNA fragments resulting from the genomic DNA breakage can be identified in situ. TUNEL assay was performed on testis of animals in control and treated groups using a standardized protocol for the "In situ Cell Death Detection Kit, Fluorescein" kit (Roche). Histological sections were deparaffinized, rehydrated, and permeabilized in Proteinase K solution (20µg / ml in 10mM Tris / HCl, pH 7.5) for 20 minutes at 37°C. After two washes in PBS for 5 minutes, the tissues were incubated with a TUNEL reaction mixture

containing 1:5 enzyme solution + labeling solution, over 60 minutes at 37 °C in a humid chamber. Then, the sections were washed one more time in PBS for 5 minutes, and finally, the slides were mounted with Fluoromount ™ Aqueous Mounting Medium (Sigma) and analyzed under fluorescence microscopy (Olympus BX60) using U-WIY filter (Olympus) for fluorescein. For the negative control, the sections were incubated only with the labeling solution, and for the positive control, the tissues were treated with recombinant DNase I (40U / ml in 50µM Tris / HCl and 1 mg/mL BSA, pH 7.5) over 10 minutes at 25 ° C before incubation with the TUNEL reaction mixture.

2.9 Transmission Electron Microscopy

Testes fragments were fixed by immersion in 4% glutaraldehyde (EMS, USA). Smaller pieces (1-2 mm thickness) were obtained and postfixed in reduced osmium (1% OsO4 and 1.5% potassium ferrocyanide in distilled water) for 90 min, dehydrated in ethanol, and embedded in Araldite epoxy resin. Ultrathin sections (60 nm thick) were obtained using a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems) and mounted on 200 mesh copper grids (Ted Pella). The ultrathin sections were stained with lead citrate (Merck, USA) and analyzed using a transmission electron microscope (Tecnai G2-12 Thermo Fisher Scientific/FEI, USA).

2.10 Hormonal Analysis

In order to perform the hormonal analysis, blood samples were collected via cardiac puncture when mice were still anesthetized before euthanasia. The plasma was separated by centrifugation at 2000rpm for 10 minutes and stored at -20ºC. Plasma testosterone concentration was measured by radioimmunoassay (RIA) using a commercial kit (Tecsa®, Belo Horizonte, MG, Brazil). The samples were processed in the automated Cobas 8000 (Roche Diagnostics Inc., Indianapolis, IN, USA) platform for direct assessment of testosterone through electrochemiluminescence assay. The testosterone coefficients of variation (CV) intra- and inter-assay were, respectively, 1.1% and 1.5%.

2.11 Statistical Analysis

All quantitative data were tested for normality and homoscedasticity of variances by the Kolmogorov-Smirnov (Dallal-Wilkinson-Lilliefor) and Bartlett tests, respectively. In the case of normal distribution, the parameters were subjected to analysis of variance (One-way ANOVA) and

averages compared by the Newman-Keuls test. For the non-parametric data, the Kruskal-Wallis test was applied for the analysis of variance, followed by the Dunns test to compare the averages. Every analysis was performed using the GraphPad Prism 5 program (GraphPad Software, Inc), and the significance level considered was $p < 0.05$.

3. RESULTS

3.1 siRNA treatment resulted in two different testicular phenotypes

After treatment, no difference in testis weight was observed in the carbon nanotubes treated animals (Fig 1B,C, F, G). For the animals treated with the complex carbon nanotubes and siRNA, two different testicular phenotypes were observed (Fig 1D-E). The treated animals with similar testicular weight were then called "treated with mild effect" (n = 6), and the ones with reduced testicular weight (~20 milligrams) were named "treated with severe effect" (n = 2) (Fig 1E-F). The GSI (Gonad somatic index) (Figure 1G) was only altered in the latter animals. Due to the reduced sampling numbers, no statistical analyzes were performed in this group.

3.2 Carbon nanotubes were identified in testes after caudal vein injection

To evaluate the siRNA's delivery in testes after applying the nanocomplex in the mice tail vein, we performed analyses on FEI Tecnai G2 F20 X-Twin transmission electron microscope (CAPI-UFMG). Confirming the local action of the nanocomplex, we identified carbon nanotubes (Fig 1H) in the interstitial compartment (in macrophages, red circle, Fig 1I) and the tubular compartment, mainly in Sertoli cells (red circles, Fig 1J-K).

3.3 The nanocomplex altered the testicular blood flow

Laser Doppler analyses showed that animals in the siRNA-treated group had greater testicular blood flow per area than animals from the control group (Fig. 1L-P). Although there was an alteration in blood flow, we found no differences in the proportion of blood vessels and lymphatic system in testis parenchyma (Fig. 1Q-R). Confirming that the altered blood flow is not related to an angiogenic stimulus, the expression of Vegf (strong angiogenic factor) (Fig. 1S) was unaltered after treatment.

3.4 Sry expression and protein are absent in the treated group with severe effects

Evaluating the *Sry* gene expression, we observed that it is absent only in the treated group with severe effects (Fig 1T). The expression of this gene was similar between the control group and the treated group with mild effects. Examining the protein presence in testis parenchyma, we observed the immunolabeling in the round and elongated spermatids (Fig 1 U-V, inserts). In the mild effect group, round spermatids were not labeled (Fig 1W), and because of the absence of germ cells, this protein was not found in the treated group with severe effects (Fig. 1X). We also evaluated the Dmrt1 and Sox9 gene expressions, and no differences were detected between controls and treated animals with mild effects (Fig 2A-B). In the animals with severe effects, the expression of Sox9 was 2.5 times higher than the values found in the other groups (Figure 2B).

3.5 The nanocomplex promoted massive germ cell sloughing from the seminiferous epithelium

Both control groups (no injection and CNTs injection) exhibited complete spermatogenesis (Fig 2C-D). It should be noted that the animals that received only carbon nanotubes had some apoptotic germ cells (Fig 2D, red arrowhead). Several seminiferous tubules lost the round and elongated spermatids in the animals with mild effects (Fig 2E, seminiferous tubule is indicated with a black asterisk). Additionally, they presented many germ cells with pyknotic nuclei (Fig 2E, red arrowhead). The testis of the animals with severe effects was mainly composed of seminiferous tubules without germ cells, a phenotype known as Sertoli cell-only syndrome (SCO) (Fig 2F, yellow asterisk). Sertoli cells were disorganized and scattered in the seminiferous epithelium, sometimes losing contact with the basal membrane of the seminiferous tubules (Fig 2F, light blue arrowhead). In addition, the seminiferous tubules did not have an evident lumen, while the intertubular compartment was relatively larger (Fig 2F, blue asterisk). Regarding epididymis' histology (Fig 2G-J), control and mild effect groups (Fig 2G-H) showed the presence of sperm cells (orange asterisks) inside the lumen of the epididymis duct (head, body and tail). Conversely, animals with mild effect presented some spermatocytes and round spermatids in the epididymal duct (Fig 2I, white asterisk). The epididymis tail lumen of animals with severe effects was filled with different types of degenerated germ cells (Fig 2J, white asterisk).

The seminiferous tubule diameter was reduced in the animals treated with the nanocomplex (Fig 2K). Evaluating the volumetric density of testis constituents (Fig 2K-P), we observed that the

tunica propria of the treated animals were enlarged compared to controls. It is essential to mention the severe reduction in the seminiferous epithelium of the treated animals with severe effects.

3.6 The nanocomplex interfered in the Sertoli cell barrier and promoted its death

The SOX9 protein (an exclusive marker of the Sertoli cell) was identified in the Sertoli cells of all groups (Fig 3A-D, red arrowheads). However, in the animals with severe effects, several Sertoli cells were not showing the presence of this protein (Fig 3D, green arrowheads). The CONNEXIN-43 protein, present in the blood-testis barrier, is continuously seen among Sertoli cells in control animals (Fig 3E-F). In the treated group with mild effect, only a few seminiferous tubules were observed with this labeling (Fig 3G), while this protein was not evidenced in the seminiferous epithelium of the animals with severe effects (Fig 3H). Although the nanocomplex altered the CONNEXIN-43 presence, it changed the expression of Claudin11 only in the animals with severe effects (Fig 3I). The treatment with the nanocomplex resulted in a diminished number of Sertoli cells per testis (Fig 3J). Some CASPASE-3 positive Sertoli cells were found in the treated animals (Fig 3K-L).

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3.7 The nanocomplex promotes germ cell apoptosis and nuclear fragmentation

The immunolabeling of CASPASE-3 (Fig 3 M-P) showed that the animals injected with only carbon nanotubes presented apoptotic germ cells (Fig 3N). However, the immunolabeling was more expressive in the treated animals with mild effects (Fig 3O, Q). This staining was not evident in the treated animals with severe effects because of the reduced quantity of germ cells (Fig 3P). Although we observed a high number of CASPASE-3 positive germ cells, the expression of this gene was not different among the groups (Fig 3R). TUNEL assay (Fig 3S-X) indicated a high nuclear fragmentation in elongated spermatids in the animals treated with the nanocomplex (Fig 3W). Although in a low frequency, it is important to mention that the animals treated only with carbon nanotubes also showed nuclear fragmentation in elongated spermatids (Fig 3V).

3.8 The nanocomplex induces Leydig cell hypertrophy

The nuclear, cytoplasmic, and total volumes of Leydig cells were increased in the animals which received the nanocomplex (Fig 4A-H). Although the occurrence of hypertrophy, the number per testis and the serum level of testosterone were not different between control and treated groups (Fig

4I-J). Concerning the Ar and Star genes (involved in the steroidogenic pathway), the major differences were identified for the animals with severe effects (Fig 4 K-L).

4. DISCUSSION

The Sry is a well-studied gene, mainly in testicular differentiation (Gubbay et al., 1990; Turner et al., 2011; Rossi et al., 1993), but its role in adult testis is not fully understood. Herein, through siRNA technology, we evaluated the consequences of a post-translational inhibition of the gene Sry. Observing the testicular phenotype of the treated animals, we hypothesized that the differential blood flow and high temperature are critical for all testicular alterations. Although more studies are necessary for the field, it is believed that an imbalance in the renin-angiotensin system (RAS) and the loss of thermoregulation can promote alterations in tight junctions, tunica propria, and cell death (Paul et al., 2006; Ely et al., 2010; Prokop et al., 2012; Milsted et al., 2010; Costa et al., 2018). For this, we will investigate the peptides and enzymes involved in the RAS and measure the testicular temperature after treatment to verify if the Sry has this fundamental vasomotor role in the testis of adult animals. Reaffirming the potential role of Sry regulating the testicular vascular system, Zwingman and colleagues (1994) observed mRNA labeling in some testicular endothelial cells through in situ hybridization.

In the present study, we observed the expression of Sry in elongated and round spermatids in the adult testis, as suggested in previous studies (Koopman et al., 1991). Zwingman et al. (1994), in a longitudinal study, identified the onset of Sry expression in animals between 21 and 28 postnatal days, a period that coincides with the emergence of round and elongated spermatids in the mice seminiferous epithelium (Auharek & France, 2010). Some authors speculated that due to the circular format of Sry's transcript, its translation would be inefficient (Hacker et al., 1995). However, knowledge from recent studies indicates that these mRNAs can, indeed, be translated, forming active proteins (Abe et al., 2015; Kristensen et al., 2019). Recently, suppression of genes activated by Sry during testicular differentiation (i.e., Sox9, Sox8, and Dmrt1) has resulted in testicular degeneration, even leading to a sex reversal in adult animals (Barrionuevo et al., 2016; Huang et al., 2017). The present findings demonstrated that Sry silencing promotes drastic changes in testicular physiology but does not lead to a sex reversal in the period under investigation. Recently, a study demonstrated that the entire Y chromosome could be replaced by overexpressing Sox9 and Eif2s3x (an important factor for the spermatogonial phase) (Ortega et al., 2015; Yamauchi et al., 2016). Although the authors achieved

offspring from the mutant mice, elongated spermatids were absent in the seminiferous epithelium, specifically the cells in which we observed a frequent SRY expression (Yamauchi et al., 2016).

One of the primary testicular changes after the treatment with siRNA occurred in germ cells, with sloughing, apoptosis, and nuclear fragmentation of spermatids. In animals with severe effects, the germ cells loss occurred more intensely, and the cross-sections of the seminiferous tubules showed a phenotype of Sertoli cell-only. Stouffs et al. (2016) reviewed the Sertoli cell-only phenotype and identified that, in most cases, sex chromosomes are affected or altered. In different phases of differentiation, germ cells were identified in the epididymis duct of the treated groups, indicating that the silencing of the Sry promoted changes in the junctions between Sertoli and germ cells (Lui & Lee, 2009; França et al., 2016). We may consider the Sry gene a cellular protective factor since its inhibition leads to germ cell death and nuclear fragmentation. Moreover, we should examine if the imbalanced RAS and loss of thermoregulation promote germ cell deaths (Chen et al., 2018; Costa et al., 2018).

After treatment with siRNA, it was possible to detect a decrease in the number of Sertoli cells and the alteration of the Sertoli cell barrier. Similarly, knockout animals for Sox9 and Sox8 also exhibited a reduction in the number of these cells per testis (Barrionuevo et al., 2016), suggesting that both genes (downstream of SRY) act as protective factors for this cell. Considering these findings, SRY may be preventing the apoptosis of Sertoli cells in our experimental model. The relative gene expression of animals with severe effects showed a greater expression of Sox9, suggesting a compensatory increase in protective factors (Barrionuevo et al., 2016). In animals with severe effects, an increase in the relative expression of Claudin11, required for the occlusion junctions between Sertoli cells, was noticed (Mruk & Cheng, 2004; França et al., 2016). This result suggests that the hematotesticular barrier may have been affected by gene silencing. Studies showed that the increase in claudin11 expression is related to defects in spermatogenesis, such as hypospermatogenesis, spermatocytary arrest, and Sertoli cell only (similar phenotype as treated animals with severe effects) (Nah et al., 2011; Mazaud-Guittot et al., 2010). This condition is consistent with the massive loss of germ cells from the seminiferous epithelium. There are also evidences that the RAS may affect the Sertoli cell barrier (Peirouvi et al., 2021; Olaniyan et al., 2020). Several studies indicate that Sry regulates RAS, stimulating the production of angiotensin II and reducing the production of angiotensin 1-7 (Paul et al., 2006; Ely et al., 2010; Prokop et al., 2012; Milsted et al., 2010). Here, again, we hypothesized that Sry silencing might promote a RAS imbalance, affecting the blood-testis barrier and causing the desquamation of germ cells from the seminiferous epithelium.

Leydig cells showed increased cell volume in treated animals with mild effects. In contrast, the total number of Leydig cells in the testis was similar between groups. A classical study evidenced that Leydig cells are hypertrophied in cases of interrupted spermatogenesis (De Kretser, 1982). The volume increase of Leydig cells may be related to a higher necessity of androgen production (Tash et al., 2002). In animals with severe effects, an increase in the expression of androgen receptors and STAR protein (critical for testosterone synthesis) was observed, indicating a compensatory strategy. It should be mentioned that imbalanced RAS can inhibit the steroidogenic activity of Leydig cells (Khanum & Dufau, 1988), and the loss of thermoregulation can induce Leydig cell hypertrophy (Costa et al., 2018). As perspectives for this study, we intend to investigate the peptides of the RAS and investigate the testicular temperature changes after the nanocomplex treatment.

5. CONCLUSION

In the present study, it was possible to conclude that the SRY protein is vital for testicular homeostasis in an adult animal. The silencing of the Sry gene resulted in testicular blood flow alteration, the release of germ cells from the seminiferous epithelium, Sertoli and germ cell apoptosis, thickening of tunica propria, and Leydig cells hypertrophy (Fig 4M-P). A better understanding of the Sry function in the testis function of adult animals is necessary, and the discovery of a new molecular signaling may help patients with fertility problems.

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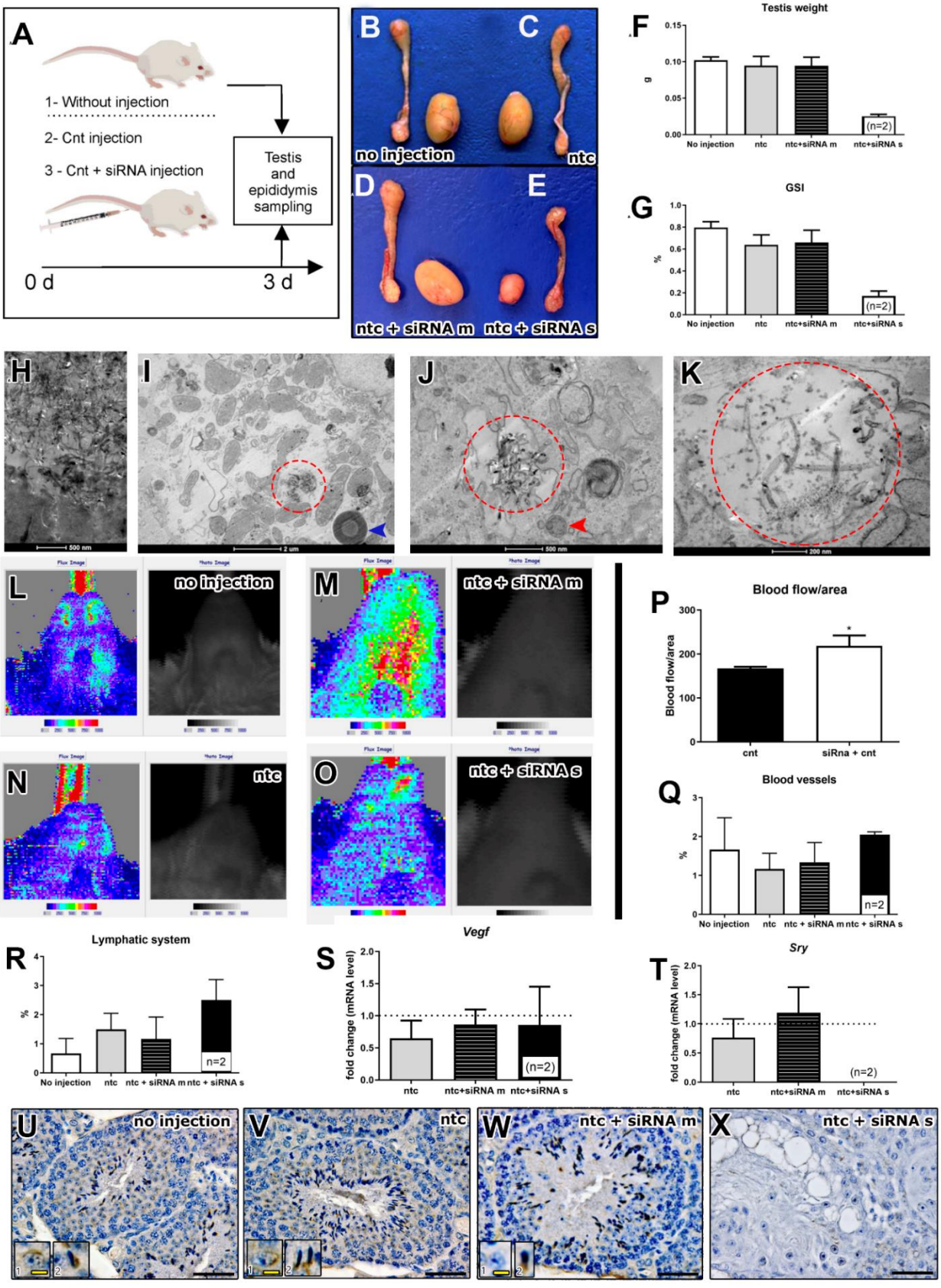
FIGURES


A
1- Without injection
2- Cnt injection
3 - Cnt + siRNA injection
Testis and epididymis sampling
0 d
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B
C
no injection
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ntc + siRNA s
F
Testis weight
g
No injection
ntc
ntc+siRNA m
ntc+siRNA s
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I
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no injection
M
ntc + siRNA m
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Blood flow/area
Blood flow/area
cnt
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No injection
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n=2
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S
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fold change (mRNA level)
T
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U
no injection
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ntc + siRNA m
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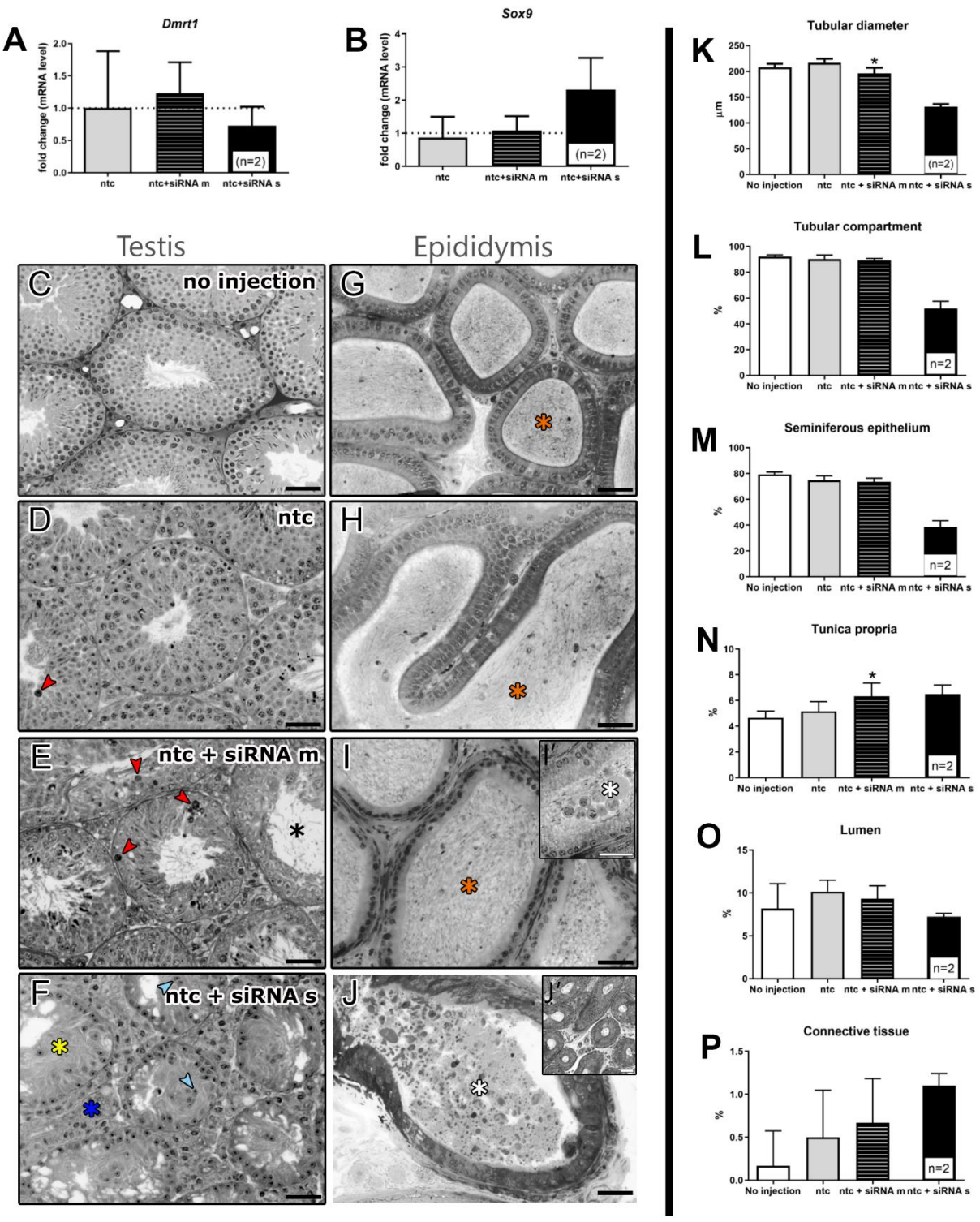
ntc + siRNA s

Figure 1. Nanocomplex injection, blood flow alteration and Sry expression. Experimental design (A). Testicles and epididymis of controls (B-C) and treated animals (D-E). The testicular weight (F) and gonadosomatic index (G) of animals after treatment (F) Transmission electronic microscopy showing the morphology of carbon nanotubes (H). Carbon nanotubes in macrophages (I, red circles). Blue arrowhead = lysosome (I). Carbon nanotubes in Sertoli cells (J-K). Red arrowhead = axoneme (J). Color map of testicular blood perfusion after treatment (L-O). Dark blue color = little or no blood perfusion; red color = maximum blood perfusion. The nanocomplex treated animals showed greater blood flow per area when compared to the control groups (P). Percentage of the testicular parenchyma occupied blood vessels (Q) and lymphatic system (R). Analysis of the relative *Vegf* and *Sry* expression (S-T). Dotted lines represent the values of the non-injected animals. Immunolocalization of SRY in the testes of adult mice (U-X). The transverse sections of seminiferous tubules demonstrate a high frequency of SRY positive round and elongated spermatids (U-V, inserts 1 and 2). Statistical analysis was performed only between groups containing 6 animals. Black scale bar = 50μm; and yellow scale bar = 5 μm.


A
Dmrt1
fold change (mRNA level)
2.0
1.5
1.0
0.5
0.0
(n=2)
ntc
ntc+siRNA m
ntc+siRNA s
B
Sox9
fold change (mRNA level)
4
3
2
1
0
(n=2)
ntc
ntc+siRNA m
ntc+siRNA s
Testis
Epididymis
C
no injection
G
D
ntc
H
E
ntc + siRNA m
I
I'
F
ntc + siRNA s
J
J'
K
Tubular diameter
µm
250
200
150
100
50
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ntc + siRNA s
L
Tubular compartment
%
100
80
60
40
20
0
n=2
No injection
ntc
ntc + siRNA m
ntc + siRNA s
M
Seminiferous epithelium
%
100
80
60
40
20
0
n=2
No injection
ntc
ntc + siRNA m
ntc + siRNA s
N
Tunica propria
%
10
8
6
4
2
0
*
n=2
No injection
ntc
ntc + siRNA m
ntc + siRNA s
O
Lumen
%
15
10
5
0
n=2
No injection
ntc
ntc + siRNA m
ntc + siRNA s
P
Connective tissue
%
1.5
1.0
0.5
0.0
n=2
No injection
ntc
ntc + siRNA m
ntc + siRNA s

Figure 2- Main testicular and epididymal morphological alterations after treatment. Dmrt1 and Sox9 expression in animals after treatment (A-B). Dotted lines represent the values of the non-injected animals. Gross histology of the testis after treatment (C-F). Degeneration of germ cells (D-E, red arrowheads). Tubule containing few germ cells (E, black asterisk). Sertoli cells only phenotype (F, yellow asterisk). Sertoli cell nuclei far from basement membrane (F, cyan arrowheads). Enlarged intertubular compartment (F, blue asterisk). Scale bar = 50 µm. Gross histology of the epididymis after treatment (G-J). The epididymal duct of controls (G-H) and treated with mild effect (I) were full of sperm (orange asterisk). Several degenerating immature germ cells were found in the tail of the epididymis of treated animals (I' and J, white asterisks). Scale bar = 50 µm. The linear measurement of the seminiferous tubule components showed a reduction in the tubular diameter of the treated animals (K). The volumetric density analyses (L-P) showed an enlargement of tunica propria (N) in treated animals.

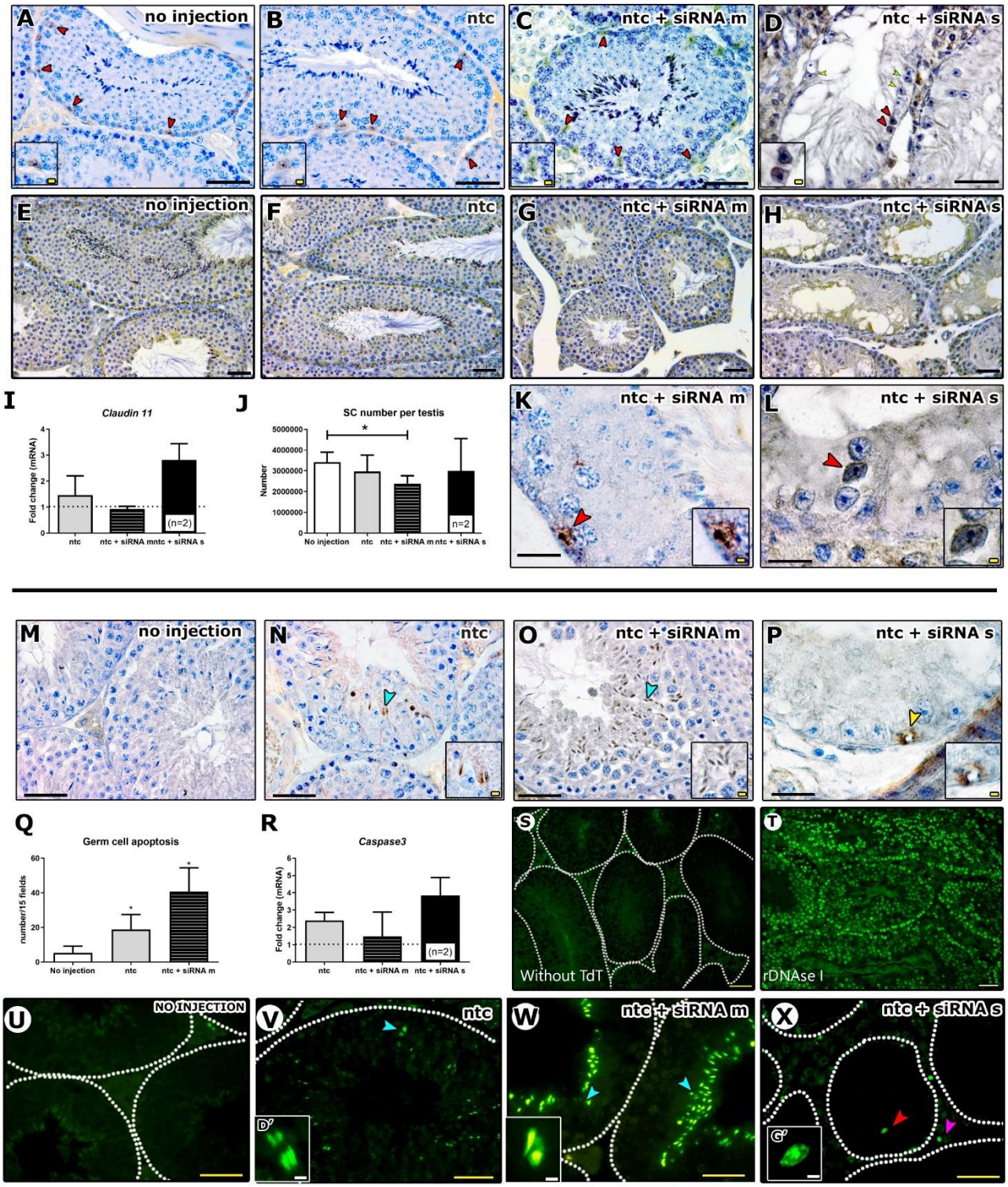
A
no injection
B
ntc
C
ntc + siRNA m
D
ntc + siRNA s
E
no injection
F
ntc
G
ntc + siRNA m
H
ntc + siRNA s
I
Claudin 11
Fold change (mRNA)
(n=2)
ntc
ntc + siRNA m
ntc + siRNA s
J
SC number per testis
Number
*
n=2
No injection
ntc
ntc + siRNA m
ntc + siRNA s
K
ntc + siRNA m
L
ntc + siRNA s
M
no injection
N
ntc
O
ntc + siRNA m
P
ntc + siRNA s
Q
Germ cell apoptosis
number/15 fields
No injection
ntc
ntc + siRNA m
R
Caspase3
Fold change (mRNA)
(n=2)
ntc
ntc + siRNA m
ntc + siRNA s
S
Without TdT
T
rDNAse I
U
NO INJECTION
V
ntc
D'
W
ntc + siRNA m
X
ntc + siRNA s
G'

Figure 3- Sertoli and germ cell alterations after treatment. SOX9 positive Sertoli cells (red arrowheads) in the different experimental groups (A-D). In severe treated animals, fewer Sertoli cells were SOX9 positive (D). Black scale bar = 50 µm; yellow scale bar = 5 µm. CONEXIN-43 immunolabeling in different experimental groups (E-H). In animals with severe effects, it was not possible to observe CONEXIN-43 immunolabeling (H). Black scale bar = 50µm. The relative expression of Claudin 11 in the different groups (I). Dotted lines represent the values of the non-injected animals. Sertoli cells number per testis after treatment (J). Caspase-3 positive Sertoli cells in treated groups (K, L - inserts). Black scale bars = 50 µm; yellow scale bar = 5 µm. Caspase-3 positive germ cells in the different experimental groups (N-O inserts = elongated spermatids; P insert = spermatogonial cell). Black scale bars = 50 µm; yellow scale bar = 5 µm. Quantification of germ cell apoptosis after treatment (Q). Relative expression of Caspase3 after treatment (R). TUNEL assay (S-X). Negative control (S, samples without TdT enzyme). Positive control (T, samples exposed to rDNAse I). TUNEL reaction in all experimental groups (U-X). Cyan arrowhead (V, W) = nuclear fragmentation of elongated spermatids. Red arrowhead and insert (X) = TUNEL positive Sertoli cell. Magenta arrowhead (X) = TUNEL positive interstitial somatic cell. Yellow scale bar = 50 µm; white scale bar = 5 µm.

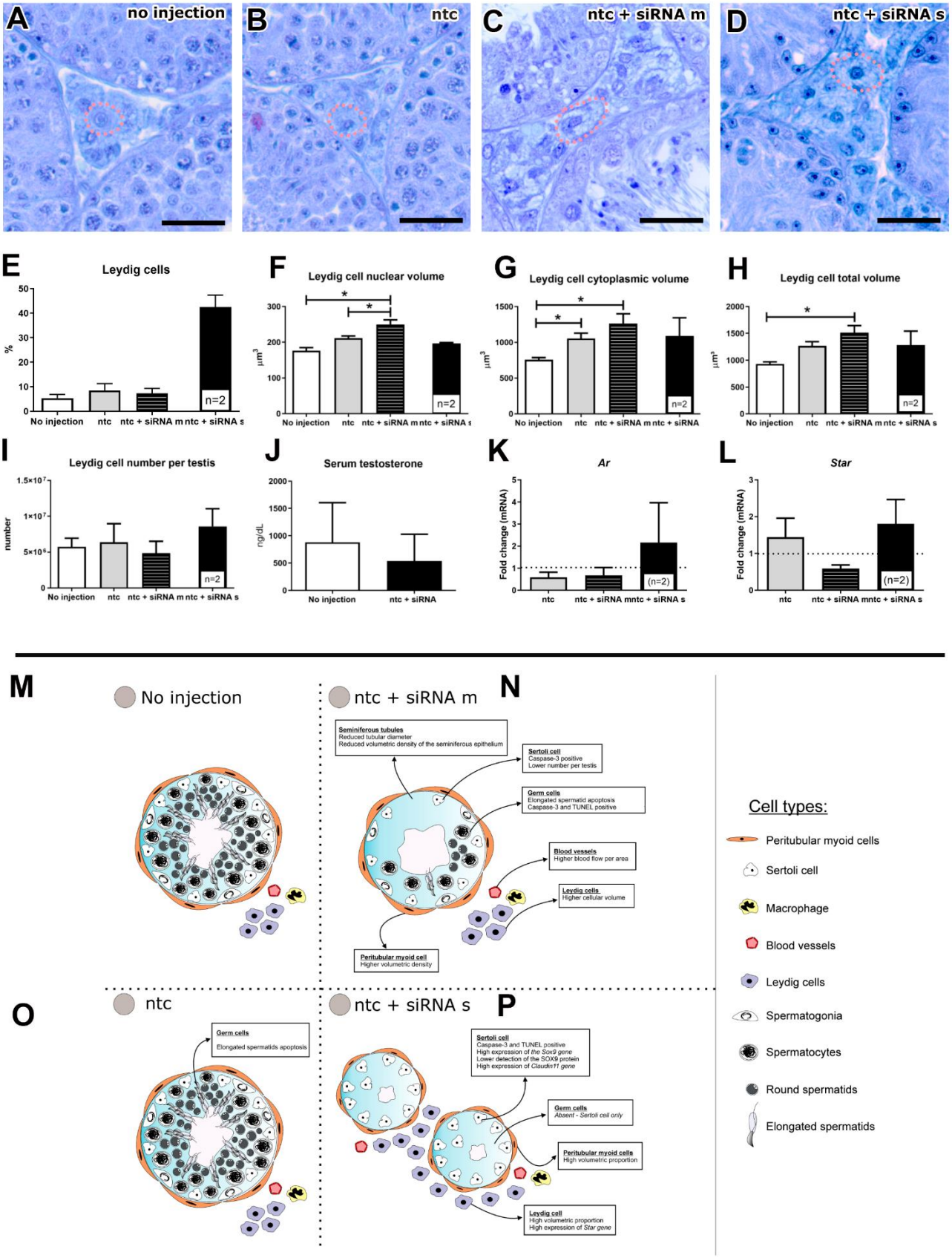
A
no injection
B
ntc
C
ntc + siRNA m
D
ntc + siRNA s
E
Leydig cells
%
n=2
No injection
ntc
ntc + siRNA m
ntc + siRNA s
F
Leydig cell nuclear volume
µm³
G
Leydig cell cytoplasmic volume
H
Leydig cell total volume
I
Leydig cell number per testis
number
J
Serum testosterone
ng/dL
K
Ar
Fold change (mRNA)
(n=2)
L
Star
M
No injection
N
ntc + siRNA m
Seminiferous tubules
Reduced tubular diameter
Reduced volumetric density of the seminiferous epithelium
Sertoli cell
Caspase-3 positive
Lower number per testis
Germ cells
Elongated spermatid apoptosis
Caspase-3 and TUNEL positive
Blood vessels
Higher blood flow per area
Leydig cells
Higher cellular volume
Peritubular myoid cell
Higher volumetric density
O
ntc
Germ cells
Elongated spermatids apoptosis
P
ntc + siRNA s
Sertoli cell
Caspase-3 and TUNEL positive
High expression of the Sox9 gene
Lower detection of the SOX9 protein
High expression of Claudin11 gene
Germ cells
Absent - Sertoli cell only
Peritubular myoid cells
High volumetric proportion
Leydig cell
High volumetric proportion
High expression of Star gene
Cell types:
Peritubular myoid cells
Sertoli cell
Macrophage
Blood vessels
Leydig cells
Spermatogonia
Spermatocytes
Round spermatids
Elongated spermatids

Figure 4 – Leydig cell alteration and summary of the findings. Histological sections showing the intertubular compartment and Leydig cell size in the different groups (A-D, red circle = Leydi cell size). Black scale bar = 50μm. The percentage of the testicular parenchyma occupied by Leydig cells (E). Nuclear volume (F), cytoplasmic volume (G), and total volume (H) of Leydig cells were increased in treated animals. The total number of Leydig cells per testis (I). Serum testosterone levels (J). Expression of the androgen receptor (K, Ar) and the acute steroidogenesis regulatory protein (L, Star) after treatment. Summary of all changes found in each group of the present study (M-P).

DISCUSSÃO INTEGRADORA DOS RESULTADOS

O *Gata-1* e o *Sry* são fatores de transcrição expressos em vários tecidos. Na literatura, cada um é bem conhecido por expressões específicas, por exemplo, o *Gata-1* no tecido hematopoiético é essencial para o desenvolvimento das células sanguíneas (Ito et al. 1993). Estudos mostram que a proteína GATA-1 alterada prejudica o desenvolvimento de basófilos e eosinófilos (Ferreira et al., 2005; Boultwood & Pellagatti, 2014; Heyworth et al., 2002; Yu et al., 2002). Já o *Sry*, é famoso pela sua expressão na crista genital, no período fetal, onde inicia toda a cascata de diferenciação testicular na maioria dos mamíferos (Sinclair et al. 1990). No testículo, os dois genes são altamente expressos, porém poucos relatos existem demonstrando suas funções na fisiologia testicular de animais adultos.

Um fato similar sobre os dois genes, é que animais knockout são inviáveis para estudos em animais adultos. Enquanto o animal knockout para GATA-1 não sobrevive devido às falhas graves no desenvolvimento do tecido hemocitopoiético (Ferreira et al., 2005), animais knockout para o SRY resultam na geração de somente fêmeas, já que a via masculina não é ativada na diferenciação sexual (Koopman et al., 1991). Nosso estudo buscou outros recursos metodológicos e tivemos acesso aos animais ΔdblGATA, um animal mutante para a proteína GATA-1. Já para os estudos com *Sry*, usamos a metodologia baseada em siRNA para inibir a tradução do RNAm do SRY. Por meio destas abordagens foi possível identificar as alterações testiculares em consequência da mutação e silenciamento gênico.

Em uma análise comparativa, podemos reforçar maior frequência de apoptose de células germinativas nos dois modelos experimentais. No animal ΔdblGATA, houve um aumento de apoptoses nos espermatócitos, causado possivelmente pela intensa diferenciação das espermatogônias-tronco (SSC). Cabe ser ressaltado que mais estudos relacionados à fisiologia das SSCs são necessários no modelo ΔdblGATA. Em relação ao silenciamento do mRNA do *Sry*, observamos a prevalência de apoptoses e fragmentação nuclear em espermátides, células que se encontraram positivamente marcadas para SRY. A investigação precisa do papel do *Sry* na espermiogênese faz-se necessária para identificar se as alterações são devidas a problemas das células germinativas ou da interação entre células de Sertoli e germinativas.

Em relação aos elementos somáticos do túbulo seminífero, não foram identificadas alterações numéricas e funcionais significativas no modelo ΔdblGATA, apesar da proteína GATA-1 estar localizada em células de Sertoli. Devemos ressaltar que alguns fatores relacionados ao “cross-talk” de células de Sertoli e células de Leydig podem estar alterados nesse sistema. Mais investigações são necessárias para detectar problemas na sinalização e comunicação das células testiculares.

Diferentemente, com o silenciamento do *Sry*, observamos o espessamento da túnica própria e redução numérica das células de Sertoli. Esse fenótipo é semelhante ao que tem sido relatado em testículos de homens acometidos pela COVID-19. A sinalização aumentada de AngiotensinaII/AT1R tem sido mencionada como a indutora do espessamento da túnica própria e responsável pela indução da morte de células testiculares (Olaniyan et al., 2020; Peirouvi et al., 2021).

Com relação ao compartimento intertubular, enfatizamos as análises em células de Leydig. No estudo do *Gata-1,* observamos aumento do número dessas células associado com a diminuição do volume celular. A expressão dos principais genes envolvidos na esteroidogênese bem como a concentração sérica de testosterona estão reduzidos no modelo ΔdblGATA. Esse desbalanço androgênico pode ter resultado em alterações na diferenciação das espermatogônias-tronco, bem como na diferenciação de espermátides alongadas. Alguns autores sugerem que os andrógenos são críticos para a síntese de GDNF pelas células peritubulares mióides (Chen et al., 2016). O aumento de espermatozoides com cabeças amorfas no fluido epididimário também pode estar relacionado com essa falha androgênica, uma vez que a espermiogênse é altamente dependente de andrógenos (De Gendt et al., 2004). Em relação ao silenciamento do gene *Sry*, acreditamos que as células de Leydig tiveram uma hipertrofia compensatória, mantendo os níveis séricos de testosterona. Devemos também ressaltar que o desbalanço do sistema renina-angiotensina (observado com a alteração do fluxo sanguíneo testicular) pode inibir as células de Leydig (Khanum & Dufau, 1988). A cultura de células de Leydig nesse modelo experimental é interessante para verificar se existe algum comprometimento das enzimas da via esteroidogênica com a inibição do RNAm do *Sry*.

Com relação ao artigo 2 (em construção), sabemos que existem algumas lacunas a serem preenchidas, principalmente em relação à participação do gene *Sry* no sistema renina-angiotensina. Sabemos que esse modelo também possui algumas limitações devido à estreita janela de atuação do nanocomplexo (3 dias). Para isso, será interessante investigar animais com sequenciais aplicações do siRNA associado ao nanotubo de carbono. Como perspectivas clínicas, pretendemos avaliar a expressão do gene *Sry* em pacientes inférteis. Ainda, o fenótipo testicular dos camundongos é semelhante ao que se tem descrito sobre a patogenia do SARS-CoV-2 no parênquima testicular. Dessa forma, pretendemos também investigar se esse gene está sendo comprometido em pacientes acometidos pela COVID-19.

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Anexos:

Cell and Tissue Research
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REGULAR ARTICLE

Activation of C–C motif chemokine receptor 2 modulates testicular macrophages number, steroidogenesis, and spermatogenesis progression

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Abstract
The monocyte chemoattractant protein 1 (MCP-1) belongs to the CC chemokine family and acts in the recruitment of C–C motif chemokine receptor 2 (CCR2)-positive immune cell types to inflammation sites. In testis, the MCP-1/CCR2 axis has been associated with the macrophage population's functional regulation, which presents significant functions supporting germ cell development. In this context, herein, we aimed to investigate the role of the chemokine receptor CCR2 in mice testicular environment and its impact on male sperm production. Using adult transgenic mice strain that had the CCR2 gene replaced by a red fluorescent protein gene, we showed a stage-dependent expression of CCR2 in type B spermatogonia and early primary spermatocytes. Several parameters related to sperm production were reduced in the absence of CCR2 protein, such as Sertoli cell efficiency, meiotic index, and overall yield of spermatogenesis. Daily sperm production decreased by almost 40%, and several damages in the seminiferous tubules were observed. Significant reduction in the expression of important genes related to the Sertoli cell function (Cnx43, Vim, Ocln, Spna2) and meiosis initiation (Stra8, Pcna, Prdm9, Msh5) occurred in comparison to controls. Also, the number of macrophages significantly decreased in the absence of CCR2 protein, along with a disturbance in Leydig cell steroidogenic activity. In summary, our results show that the non-activation of the MCP-1/CCR2 axis disturbs the testicular homeostasis, interfering in macrophage population, meiosis initiation, blood–testis barrier function, and androgen synthesis, leading to the malfunction of seminiferous tubules, decreased testosterone levels, defective sperm production, and lower fertility index.

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Depositante 1 de 2

Nome ou Razão Social: UNIVERSIDADE FEDERAL DE MINAS GERAIS

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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 29/04/2020 às 17:16, Petição 870200053488

Depositante 2 de 2

Nome ou Razão Social: FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DE MINAS GERAIS - FAPEMIG
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Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)
Título da Invenção ou Modelo de Utilidade (54): COMPOSIÇÃO DE NANOPARTÍCULAS SUPERPARAMAGNÉTICAS PARA APLICAÇÃO INTRATESTICULAR E USO
Resumo: A presente tecnologia trata uma composição de nanopartículas superparamagnéticas para aplicação intratesticular, compreendendo nanopartículas de óxido de ferro funcionalizadas com citrato de sódio, e
de seu uso para esterilização de mamíferos machos por indução de hipertermia, através de aplicação de um campo magnético alternado. O uso da composição promove a depleção das células de Leydig, bem como da espermatogênese em mamíferos.
Figura a publicar: 3

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE MORFOLOGIA

DECLARAÇÃO

Declaro, para os devidos fins e a pedido do interessado, que **MATHEUS FELIPE FONSECA GONÇALVES**, CPF **101.838.116-31** atua como **PROFESSOR VOLUNTÁRIO ou SUBSTITUTO** junto ao DEPARTAMENTO DE MORFOLOGIA do INSTITUTO DE CIÊNCIAS BIOLÓGICAS, desde 1o de agosto de 2018 até o momento.

Atuou como **PROFESSOR VOLUNTÁRIO** no ano de 2018 e no 1º semestre de 2019 na disciplina **ANATOMIA VETERINÁRIA I - MOF047**, assumindo Aulas Práticas e Teóricas, onde cumpriu a carga horária de **6** horas semanais, totalizando **90** horas. No 2º semestre de 2019, atuou como **PROFESSOR SUBSTITUTO** nas disciplinas: **ANATOMIA VETERINÁRIA I - MOF047** e **ANATOMIA VETERINÁRIA II - MOF048**, lecionando Aulas Práticas e Teóricas, onde cumpriu carga horária de **13,0** horas semanais, totalizando **195,00** horas. Em seguida atuou no ano de 2020 no 1º semestre, na disciplina **ANATOMIA VETERINÁRIA I - MOF047**, cumprindo carga horária de **3** horas semanais, totalizando **45** horas. Como **PROFESSOR VOLUNTÁRIO** está atuando no 2º semestre de 2021 na disciplina **ANATOMIA VETERINÁRIA I - MOF047**, assumindo Aulas Práticas e Teóricas, cumprindo carga horária de **6** horas semanais, e totalizando **90** horas.

Média semanal de carga horária: **7** horas (por ano).

Média total de carga horária: **105** horas (por ano).

Belo Horizonte, 12 de novembro de 2021.

Profa. Annamaria Ravara Vago
Chefe *Pro-Tempore* do Departamento de Morfologia
***Instituto de Ciências Biológicas* da UFMG**
Portaria N° 4.035 de 31/07/2020.

Documento assinado eletronicamente por **Annamaria Ravara Vago**, **Chefe de departamento**, em 12/11/2021, às 16:48, conforme horário oficial de Brasília, com fundamento no art. 5º do Decreto nº 10.543, de 13 de novembro de 2020.

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V Simpósio de Integração dos Programas de Pós-Graduação em Biologia Celular
VIII Simpósio de Biologia Celular da UFMG- Profa. Cleida Aparecida de Oliveira

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CERTIFICADO

Certificamos que o trabalho intitulado "**ANÁLISE MORFOFUNCIONAL DO TESTÍCULO APÓS O SILENCIAMENTO PÓS-TRANSCRICIONAL DO GENE SRY**" de autoria de **M.F.F. Gonçalves, S.M.S.N. Lacerda, N.L.M. Lara, F. Alves, F.O. Vieira, A.S. Martins, L.B. Prado, L.S. Barcelos, A.O. Carmo, E. Kalapothakis, G.M.J. Costa** , foi apresentado na forma de pôster durante o V Simpósio de Integração dos Programas de Pós-Graduação em Biologia Celular e VIII Simpósio de Biologia Celular – Profª Cleida Aparecida de Oliveira, realizado na UFMG entre os dias 18 e 22 de setembro de 2017.

Belo Horizonte, 22 de setembro de 2017.

Wiviane Alves Assis
Presidente do V SIBC- UFMG 2017

Fernanda Radicchi Campos Lobato de Almeida
SubCoordenadora da PPG Biologia Celular UFMG

V Simpósio de Integração dos Programas de Pós-Graduação em Biologia Celular
VIII Simpósio de Biologia Celular da UFMG- Profa. Cleida Aparecida de Oliveira

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CERTIFICADO

Certificamos que **Matheus Felipe Fonseca Gonçalves** participou do V Simpósio de Integração dos Programas de Pós-graduação em Biologia Celular e VIII Simpósio de Biologia Celular da UFMG – Profª Cleida Aparecida de Oliveira, realizado na UFMG entre os dias 18 e 22 de setembro de 2017, com carga horária total de 35 horas.

Belo Horizonte, 22 de setembro de 2017

Wiviane Alves Assis
Presidente do V SIBC- UFMG 2017

Profª Fernanda Radicchi Campos Lobato de Almeida
Subcoordenadora do PPG Biologia Celular/UFMG

V Simpósio de Integração dos Programas de Pós-Graduação em Biologia Celular

VIII Simpósio de Biologia Celular da UFMG- Profa. Cleida Aparecida de Oliveira

UFMG UFV UFU UFSJ

CERTIFICADO

Certificamos que **Matheus Felipe Fonseca Gonçalves** participou do minicurso **"Using Powerpoint without Powerpoint using you"**, realizado durante o V Simpósio de Integração dos Programas de Pós-graduação em Biologia Celular e VIII Simpósio de Biologia Celular da UFMG – Profª Cleida Aparecida de Oliveira, no dia 19 de setembro de 2017, com carga horária total de 2 horas.

Belo Horizonte, 22 de setembro de 2017

Wiviane Alves Assis
Presidente do V SIBC- UFMG 2017

Profª Fernanda Radicchi Campos Lobato de Almeida
Subcoordenadora do PPG Biologia Celular/UFMG

CERTIFICADO

Certificamos que **Matheus Felipe Fonseca Gonçalves** apresentou a palestra intitulada "Como gerar o interesse do aluno" durante o evento **Proseando sobre o estágio didático e o ensino no Departamento de Morfologia** realizado no Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais no dia 18 de maio de 2018.

Coordenadora do Estágio Didático
Dra. Profa. Cleida Aparecida de Oliveira

Coordenadora do Programa de Pós-graduação em Biologia Celular
Dra. Profa. Erika Cristina Jorge

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INSTITUTO DE CIÊNCIAS BIOLÓGICAS

DEPARTAMENTO DE MORFOLOGIA

DECLARAÇÃO

Declaro, para os devidos fins, que **MATHEUS FELIPE FONSECA GONÇALVES**, CPF: **101.838.116-31**, atuou como **PROFESSOR VOLUNTÁRIO** junto ao DEPARTAMENTO DE MORFOLOGIA, do INSTITUTO DE CIÊNCIAS BIOLÓGICAS, no segundo semestre letivo de 2016 e nos primeiro e segundo semestres letivos de 2017, nas disciplinas **ANATOMIA VETERINÁRIA I e II**, ministradas para o curso de MEDICINA VETERINÁRIA, com carga horária de 04 horas semanais, totalizando 180 horas.

Belo Horizonte, 02 de fevereiro de 2018.

Profª. Tânia Mara Segatelli
Chefe Depto. Morfologia
Portaria nº. 6.883 de 26/10/2017

Profª Drª Tânia Mara Segatelli
Chefe do Departamento de Morfologia
ICB/UFMG

Escola de Veterinária
UFMG

O Centro de Extensão da Escola de Veterinária da Universidade Federal de Minas Gerais confere o presente certificado a **MATHEUS FELIPE FONSECA GONÇALVES** por ter Ministrado a palestra "*Neuroanatomia aplicada*", no *I SIMPÓSIO DE NEUROIMAGEM EM MEDICINA VETERINÁRIA*, realizado no Instituto de Ciências Biológicas-ICB/UFMG, em 31 de outubro de 2019, totalizando carga horária de 1(uma) hora

Belo Horizonte, 31 de outubro de 2019.

Profa.Zélia Inês Portela Lobato
Diretora da Escola de Veterinária da UFMG

Prof. Matheus Anchieta Ramirez
Coordenador do Cenex-EV/UFMG

ESCOLA DE VETERINÁRIA – UFMG – Centro de Extensão

Certificado Nº 20.557

CERTIFICADO

Certificamos que Matheus Felipe Fonseca Gonçalves ministrou o minicurso **"Neuroanatomia Veterinária"** no **I Colóquio Técnico e Cientifico da Medicina Veterinária do UniBH**, realizado no dia 23 de maio de 2018, com duração de 3 horas.

Belo Horizonte, 23 de maio de 2018

BRUNO ANTUNES SOARES
Coordenador do Curso de Medicina Veterinária

PRHISCYLLA SADANÃ PIRES
Profa. Adjunta de Medicina Veterinária
Núcleo Docente Estruturante

I Colóquio Técnico & Científico
Medicina Veterinária UniBH

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Certificamos que, **Matheus Felipe Fonseca Gonçalves** participou do SAÚDE – Noite T1 programa de Formação e Capacitação Docente, promovido pela ĂNIMA EDUCAÇÃO realizado em fevereiro de 2019, com carga horária de 12 horas de atividade.

Belo Horizonte, 06 de setembro de 2019.

Diretoria de Formação e Capacitação Docente – ĂNIMA

Reitor

CERTIFICADO

Certifico que MATHEUS FELIPE FONSECA GONÇALVES ministrou a PALESTRA: ANATOMIA DO SISTEMA DIGESTÓRIO DO EQUINO, realizada no dia 12 de abril de 2021 com a duração de duas horas.

Cynthia Gieseke Meniconi Alenquer
Professora Organizadora

Bruno Antunes Soares
Diretor de Campus

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CERTIFICADO

A Red Latinoamericana de Reproducción Asistida (REDLARA)
emite o presente certificado ao

Professor Matheus Felipe Fonseca Gonçalves

por sua colaboração ao Módulo Espermatogênese realizada para o Curso Embriologia Clínica do PEC Online.

13 de maio de 2021

Maria do Carmo Borges de Souza
Presidente

Cláudia Petersen
Coordenadora PEC Online Embriologia

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MATHEUS FELIPE FONSECA GONÇALVES

Ministrou a PALESTRA: FUNDAMENTOS BÁSICOS DA OFTALMOLOGIA, realizada no dia 02 de julho de 2021, pelo Centro Universitário Una com a duração de duas horas.

Belo Horizonte, 05 de julho de 2021

Bruno Antunes Soares
Diretor de Campus

Cynthia Gieseke Meniconi
Professora Organizadora

Pós-Graduação Genética UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOLOGIA GERAL

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

Certificamos que, Matheus Felipe Fonseca Gonçalves, assistiu o Seminário Internacional intitulado **"ANIMAL SEX DETERMINATION BY GENES, CHROMOSOMES AND THE ENVIRONMENT"**, apresentado pela Professora Dra. Jennifer Marshall Graves, Fellow of the Australian Academy of Science, no Programa de Pós-Graduação em Genética, ICB/UFMG, em 24/05/2019, com duração de 3 horas.

Belo Horizonte, 27 (vinte e sete) de Maio de 2019.

Prof. Dr. Evanguedes Kalapothakis
Coordenador do Programa de Pós-graduação em Genética