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CAMPUS JABOTICABAL

EFEITOS DA GONADOTROFINA CORIÔNICA HUMANA NOS PARÂMETROS REPRODUTIVOS DE BODES DURANTE A CONTRA ESTAÇÃO REPRODUTIVA

Gabriel Brun Vergani

Médico Veterinário

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DADOS CURRICULARES DO AUTOR

GABRIEL BRUN VERGANI – nascido em Toledo – PR, aos 22 dias do mês de novembro de 1994. Graduou-se em Medicina Veterinária, na Universidade Federal do Paraná – UFPR Setor Palotina - PR, em 2018. Cursou Pós-graduação em Medicina Veterinária, nível de Mestrado e área de concentração de Reprodução Animal, na Faculdade de Ciências Agrárias e Veterinárias, da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP Câmpus de Jaboticabal-SP, de março de 2018 a julho de 2020, sob orientação da Profa. Dra. Maria Emilia Franco Oliveira e coorientação do Prof. Dr. Jeferson Ferreira da Fonseca, como bolsista do CNPQ. Ingressou no curso de Pós-graduação em Medicina Veterinária, nível de Doutorado e área de concentração de Reprodução Animal, na Faculdade de Ciências Agrárias e Veterinárias, da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP Câmpus de Jaboticabal-SP, em agosto de 2020, sob orientação da Profa. Dra. Maria Emilia Franco Oliveira, como bolsista da CAPES.

“O progresso é impossível sem mudanças, e aqueles que não conseguem mudar as suas mentes não conseguem mudar nada”

George Bernard Shaw

Dedico esse trabalho aos meus pais, Rafael Bergamaschi Vergani e Vania Maria Brun Vergani. Amo vocês!

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CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Embrapa Gado de Leite

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Programa de Melhoramento Genético de Caprinos Leiteiros (Capragene) - Fase 2 (20.19.01.004.00.00) □ Estratégias de para a elevação da taxa de gestação de cabras acasaladas naturalmente ou inseminadas artificialmente durante diferentes estações do ano", protocolada sob o CEUA nº 5755150721, sob a responsabilidade de **Jeferson Ferreira da Fonseca** *e equipe; Gabriel Brun Vergani; Juliana Nascimento Duarte Rodrigues; Luiz Gustavo Bruno Siqueira; Maria Emília Franco Oliveira; Tarcilla Dutra Barbosa Caldas; Antônio Cândido de Cerqueira Leite Ribeiro* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Embrapa Gado de Leite (CEUA/EGL) na reunião de 07/10/2021.

We certify that the proposal "Dairy Goats Genetic Improvement Program (Capragene) - Phase 2 (20.19.01.004.00.00) □ Strategies to increase the pregnancy rate of naturally mated or artificially inseminated goats during different seasons", utilizing 208 Caprines (30 males and 178 females), protocol number CEUA 5755150721, under the responsibility of **Jeferson Ferreira da Fonseca** *and team; Gabriel Brun Vergani; Juliana Nascimento Duarte Rodrigues; Luiz Gustavo Bruno Siqueira; Maria Emília Franco Oliveira; Tarcilla Dutra Barbosa Caldas; Antônio Cândido de Cerqueira Leite Ribeiro* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Embrapa Gado de Leite Corporate (CEUA/EGL) in the meeting of 10/07/2021.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 10/2021 a 10/2023 Área: Núcleo Produção E Bem Estar Animal

Origem:	Campo Experimental José Henrique Bruschi						
Espécie:	Caprinos	sexo:	Fêmeas	idade:	08 a 84 meses	N:	178
Linhagem:	Leiteira			Peso:	30 a 80 kg		
Origem:	Campo Experimental José Henrique Bruschi						
Espécie:	Caprinos	sexo:	Machos	idade:	18 a 84 meses	N:	30
Linhagem:	Leiteira			Peso:	45 a 80 kg		

Local do experimento: Campo Experimental José Henrique Bruschi / Embrapa Gado de Leite / Coronel Pacheco - MG

Juiz de Fora, 08 de outubro de 2021

Dra. Naiara Zoccal Saraiva
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Virginia de Souza Columbiano Barbosa
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Embrapa Gado de Leite

EFEITOS DA GONADOTROFINA CORIÔNICA HUMANA NOS PARÂMETROS REPRODUTIVOS DE BODES DURANTE A CONTRA ESTAÇÃO REPRODUTIVA

RESUMO – O estudo tem como objetivo investigar se o uso da administração repetida de hCG durante a contra estação reprodutiva melhora as concentrações de testosterona, os parâmetros escrotais/testiculares e a qualidade do sêmen em caprinos machos. Vinte bodes foram divididos em dois grupos (G-Controle, n = 10; e G-hCG, n = 10) para receber quatro administrações (im) de 1 mL de solução salina ou 300 UI de hCG, respectivamente, em intervalos de 14 dias. (D0, D14, D28 e D42). Nos dias D-7, D0, D14, D28, D42 e D56 foram coletadas amostras de sêmen com vagina artificial para avaliação antes e após a criopreservação. Os parâmetros biométricos foram avaliados um dia antes da coleta de sêmen. Amostras de sangue foram obtidas nos dias de coleta de sêmen e nos dias D1, D7, D15, D21, D29, D35, D43 e D49 para dosagem hormonal. Os dados foram analisados com o software SAS, utilizando modelos mistos com medidas repetidas no tempo, sendo considerado o tratamento e a interação entre tratamento e tempo como efeitos principais, o D-7 foi utilizado como covariável. As concentrações séricas de testosterona foram maiores (P = 0,0005) no G-hCG (9,6 ± 0,6 ng/mL) em comparação com o G-Control (6,3 ± 0,6 ng/mL). Não houve efeito do tratamento (P > 0,05) para a biometria escrotal/testicular, parâmetros do sêmen fresco, bem como na cinética do sêmen pós-descongelamento. Maior número de células vivas com alto potencial de membrana mitocondrial (MMP) foram observadas (P < 0,05) no último dia de avaliação (D56) para os animais do G-hCG (49,0 ± 6,6 %) em comparação aos animais G-Control (26,5 ± 7,4 %). Além disso, o status de capacitação espermática foi observado interação entre os efeitos sendo observado maior valor (P = 0.0001) no D56 para os machos G-hCG (36,5 ± 8,8 %) em comparação ao G-Control (11,5 ± 9,0 %). Em conclusão, quatro administrações de 300 UI de hCG em intervalos de 14 dias durante a contra estação reprodutiva são eficazes para melhorar as concentrações séricas de testosterona e promover maior estado de capacitação espermática e espermatozoides vivos com alto potencial de membrana mitocondrial em caprinos machos.

Palavras-chave: Gonadotrofina coriônica humana, potencial de membrana mitocondrial, integridade da membrana plasmática, criopreservação de sêmen, cinética espermática, status de capacitação espermática.

EFFECTS OF HUMAN CHORIONIC GONADOTROPHIN ON THE REPRODUCTIVE PARAMETERS OF MALE GOATS DURING THE NON-REPRODUCTIVE SEASON

ABSTRACT – The study aims to investigate if the use of repeated administration of hCG during the non-breading season improves testosterone concentrations, scrotal/testicular parameters, and sperm quality in male goats. Twenty bucks are divided into two groups (G-Control, n = 10; and G-hCG, n = 10) to receive four administrations (im) of 1 mL of saline or 300 IU of hCG, respectively, at 14-day intervals (D0, D14, D28, and D42). On D-7, D0, D14, D28, D42, and D56 semen samples with artificial vagina were collected to be evaluated before and after cryopreservation. Biometric parameters were evaluated the day before the semen collection. Blood samples were obtained on the days of semen collection and on the days D1, D7, D15, D21, D29, D35, D43, and D49 to hormonal dosage. The data was analyzed with the SAS software, using mixed models with repeated measures over time, considering treatment and the interaction between treatment and time as main effects, D-7 was used as a covariate. Serum testosterone concentrations were higher (P = 0.0005) in G-hCG (9.6 ± 0.6 ng/mL) compared with G-Control (6.3 ± 0.6 ng/mL). There were no treatment effects (P > 0.05) for scrotal/testicular biometry, fresh semen parameters as well as post-thawing semen kinetic characteristics. High mitochondrial membrane potential (MMP) cells were observed (P < 0.05) on the last day of evaluation (D56) for G-hCG animals (49.0 ± 6.6 %) compared to G-Control ones (26.5 ± 7.4 %). Furthermore, the sperm capacitation status was observed an interaction between the effects, with a higher value (P = 0.0001) observed on D56 for G-hCG males (36.5 ± 8.8 %) compared to G-Control (11.5 ± 9.0 %). In conclusion, four administrations of 300 IU of hCG at 14-day intervals during the non-breeding season are effective in improving the serum testosterone concentrations and promoting higher sperm capacitation status and live sperm with high mitochondrial membrane potential in male goats.

Keywords: Human Chorionic Gonadotropin, mitochondrial membrane potential, plasma membrane integrity, semen cryopreservation, sperm kinetics, sperm capacitation status.

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CAPÍTULO 1 – Considerações Gerais

1. INTRODUÇÃO

Pequenos ruminantes apresentam uma característica denominada de ciclo reprodutivo sazonal, demonstrando esse comportamento em locais onde há alterações da duração do dia ao longo do ano. Assim, com esse ciclo reprodutivo, são capazes de manifestar períodos aptos à reprodução em momentos do ano que permitirão que ao nascimento de seus descendentes, sejam épocas com melhor disponibilidade de alimento e temperatura (Goldman, 2001). Dentre as alternativas para reduzir esses efeitos da sazonalidade, temos a suplementação de melatonina, manejo de luz artificial e aplicações de gonadotrofinas exógenas, possibilitando diferentes resultados e durações de protocolos para melhorar a qualidade seminal e libido dos machos durante a contra estação.

Os estudos relacionados a compreender as alterações reprodutiva em machos durante a contra estação são essenciais para o manejo e eficiência zootécnica da produção a campo. Ao se conhecer o processo reprodutivo sazonal de uma determinada espécie, pode-se fazer a correta escolha do reprodutor para estação de monta de um rebanho, além de identificar as melhores técnicas e períodos para a coleta e criopreservação do sêmen utilizado nas biotecnologias reprodutivas. Portanto, ao se compreender as variáveis reprodutivas dos animais sazonais podem ser criadas alternativas para diminuir ou eliminar os períodos de montas restritas, e aumentar a produção de produtos de origem animal/ano.

A hipótese do presente estudo é de que a administração seriada de hCG em machos caprinos durante a contra estação reprodutiva induz aumento nas concentrações séricas de testosterona e, consequentemente, induz efeitos positivos sobre as características biométricas testiculares/escrotais e qualidade seminal. O objetivo do presente estudo é avaliar a utilização de quatro doses de hCG (300 UI), em intervalos de 14 dias, em machos caprinos adultos durante a contra estação reprodutiva nas características biométricas escrotais/testiculares,

concentrações séricas de testosterona e características do sêmen fresco e pós criopreservação.

2. REVISÃO DE LITERATURA

2.1 Os efeitos da sazonalidade na fisiologia reprodutiva do reprodutor caprino

As características sazonais de reprodução se expressam com maior intensidade naqueles animais com origem em regiões subtropicais, no hemisfério norte ou sul, com latitudes superiores a 35° Norte ou 35° Sul (Avdi, et al., 2004; Sarlós, et al., 2013). O principal fator afetando a atividade reprodutiva nos animais é o ciclo anual do fotoperíodo, com as alterações da disponibilidade de horas luz diárias, enquanto o clima (umidade e temperatura), o estado nutricional do animal, presença ou ausência de alimento e a interação social, são moduladores da sazonalidade reprodutiva (Milczewski, 2008). Em consequência destes efeitos anuais relacionados ao fotoperíodo, ocorrem alterações na fisiologia reprodutiva destes animais, devido às supressões no eixo hipotálamo-hipófise-gonadal, devido à redução na produção de melatonina durante a contra estação, momento em que os dias são mais longos (primavera-verão), já a estação reprodutiva na qual os dias são mais curtos (outono-inverno) (Sousa et al., 2008).

Devido à redução na produção de melatonina no início do período de transição e posterior período de contra estação reprodutiva, algumas alterações hormonais, físicas e comportamentais são observadas nos machos das espécies caprina e ovina, principalmente a redução na regulação da síntese de GnRH que irá diminuir a atividade do eixo hipotálamo hipófise gonadal (Figura 1). A melatonina é um hormônio essencial para que haja a síntese e liberação dos hormônios gonadais. Este hormônio envia sinais que regulam a sazonalidade da reprodução, nos machos, exerce efeitos específicos sobre o hipotálamo, desencadeando, por feedback positivo, a liberação pulsátil de gonadotrofinas, estabelecendo assim o controle sobre a atividade sexual (Harman & Serpek, 2022).

Diferente da fêmea onde há o período de anestro, no qual não ocorre a principal etapa do ciclo estral que é a ovulação, o macho não para sua produção de espermatozoides no momento de contra estação, continuando assim a

produção mesmo com níveis baixos de testosterona (Pelletier & Almeida, 1987). A maioria das raças de caprinos e ovinos que estão em condições subtropicais de criação, sofrem algum nível de alteração na produção de melatonina ao longo do ano e sofrem alterações principalmente no comportamento sexual.

As principais alterações observadas durante a contra estação nos machos caprinos são as mudanças no comportamento sexual (libido), nas dimensões testiculares (massa e volume), na espermatogênese e secreção hormonal de testosterona e demais hormônios gonadais (Kafi, et al., 2004; Zamiri & Khodaei, 2005). A espermatogênese ocorre em etapas, na primeira ocorre a espermatocitogênse (fase de proliferação), que consiste em divisões mitóticas que ocorrem nas espermatogônias gerando um alto número de espermatogônias tipo B; fase denominada de meiótica, que envolve os espermatócitos primários e secundários, com a produção de espermátides haploides; em segundo, a fase de espermiogênese (fase de diferenciação), neste momento não ocorrem mais divisões celulares como na etapa anterior, agora a espermátide haploide, passa por uma série de transformações que resulta na produção do espermatozoide, contendo uma cabeça, uma peça intermediaria e um flagelo. Para que ocorram estas diferenciações as células são dependentes de níveis ideais de testosterona, principalmente o desenvolvimento das espermátides alongadas a partir das arredondadas (Odonnel et al. 1994). A testosterona está diretamente envolvida em diversos processos ligados a síntese e manutenção da viabilidade espermática e apresenta flutuações nas concentrações plasmáticas ao longo do ano (Santiago-Moreno et al.,2005; Todini et al.,2007).

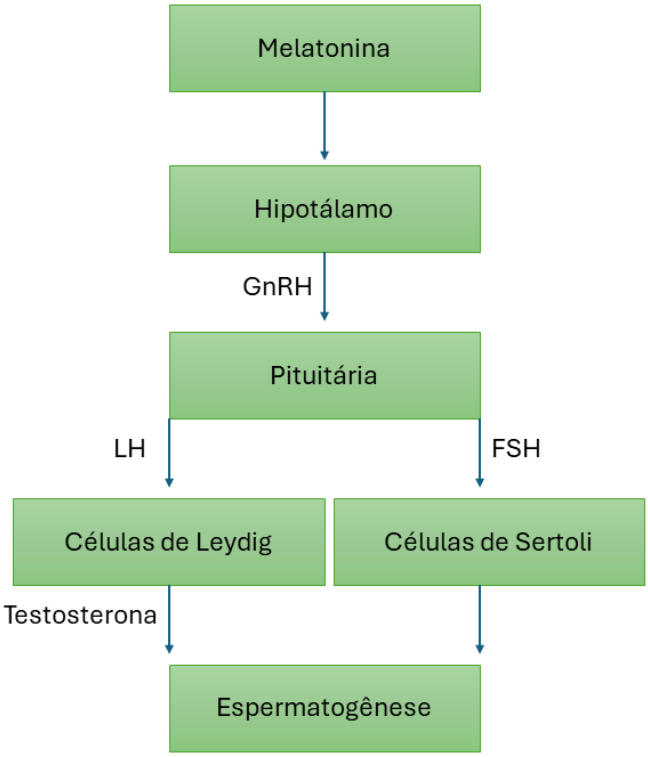


Figura 1. Representação esquemática das inter-relações entre o hipotálamo, a hipófise e os testículos. Liberação de gonadotrofina hormônio (GnRH) estimula a liberação das gonadotrofinas hipofisárias hormônio luteinizante (LH) e hormônio folículo-estimulante (FSH). O hormônio luteinizante atua na espermatogênese via testosterona produzido pelas células de Leydig. FSH atua nas células de Sertoli que atuam diretamente na espermatogênese.

2.2 Síntese de testosterona e sazonalidade

A testosterona é necessária para a espermatogênese nos testículos e posterior maturação nos epidídimos, para manter a função das glândulas acessórias, que são essenciais para a produção de um plasma seminal rico que fara a manutenção da viabilidade espermática após a ejaculação e para o desenvolvimento de características sexuais secundárias masculinas (Noakes et al., 2001). A principal fonte de testosterona são as células de Leydig a qual é controlada principalmente pela secreção em picos e pulsante ao hormônio hipofisário LH (Zirkin e Papadopoulos, 2018). O hormônio LH secretado pela glândula pituitária em resposta as liberações de GnRH do hipotálamo, dá início a formação de esteroides pela ligação ao receptor de LH presente nas células de Leydig (LHR). Através do acoplamento à proteína G, ocorre o estímulo à produção de adenosina 3',5'-monofosfato cíclico (cAMP ou AMP cíclico) pelas células de Leydig. Por sua vez, o AMP cíclico, estimula a translocação de colesterol do meio intracelular até as mitocôndrias, etapa determinante da produção de esteroides. Em seguida ocorre a formação de pregnenolona pelas mitocôndrias a partir da conversão do colesterol obtido, e finalmente este precursor será transformado em testosterona pelo retículo endoplasmático liso (Zirkin e Papadopoulos, 2018) (Figura 2).

Dentre as funções reprodutivas da testosterona, podemos citar a regulação das células de Sertoli estimulando os estádios finais da espermatogênese, estímulo ao desenvolvimento durante a fase embrionária do macho, desenvolvimento de órgãos reprodutivos secundários, manutenção da libido e a manutenção das características sexuais secundárias do macho (Gardner & Hafez, 2004). Um estudo observou que existe uma forte relação ($r = 0.71$), entre a sazonalidade e a síntese de testosterona em carneiros, sendo na estação reprodutiva a maior síntese deste hormônio (Sarlós et al., 2013). Porém como citado anteriormente, a sazonalidade é um fator que afeta a síntese da melatonina, mas existem outros moduladores intrínsecos e extrínsecos aos animais que podem também estar intensificando ou não os efeitos do fotoperíodo na fisiologia reprodutiva destes machos (Barbosa et al., 1999).

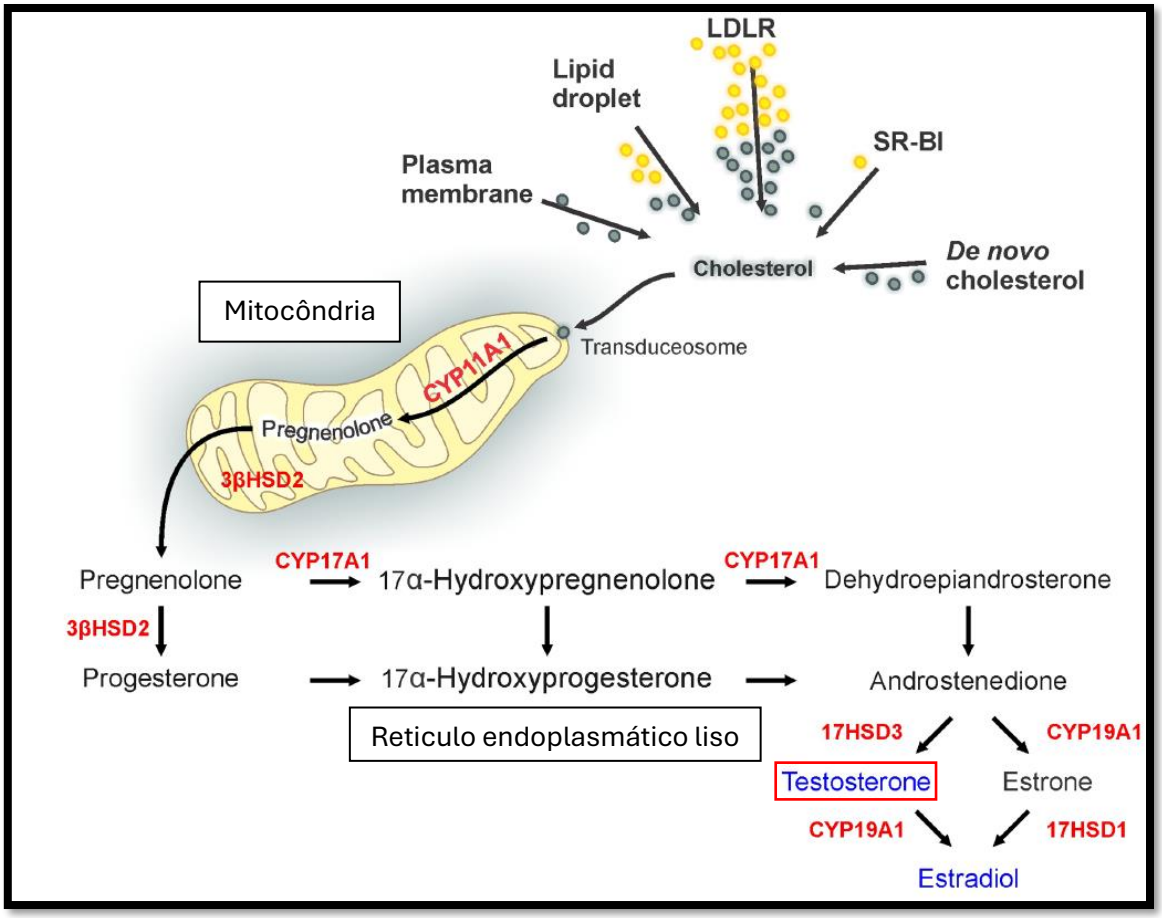


Figura 2: Esquematização da síntese de esteroides nos testículos. Fonte: adaptado de (Zirkin e Papadopoulos, 2018).

As células de Sertoli são alvos primários do FSH e da testosterona, que são hormônios crucias para a manutenção da espermatogênese normal. Durante a contra estação, estes dois hormônios estão em níveis reduzidos nos bodes em comparação com a estação reprodutiva (Xi et al., 2021). Em veados já foi observado que o FSH e a testosterona são importantes para a proliferação das espermatogônias e produção de espermatozoides durante a estação reprodutiva (Roelants et al., 2002). O FSH desempenha um papel fundamental na produção e secreção da proteína carreadora de andrógeno (ABP), fator neurotrófico derivado de linhagem celular glial (GDNF) e fator de células troncos (SCF) através do seu receptor nas células de Sertoli (FSHR) (Bhattacharya et al., 2019). O GDNF e SCF regulam a diferenciação e proliferação das células germinativas, enquanto o ABP estimula e promove a espermatogênese elevando as concentrações de testosterona dentro dos túbulos seminíferos. Importante

observar que os níveis de expressão da ABP, GDNF, SCF são significativamente maiores durante a estação reprodutiva em comparação com a contra estação em bodes leiteiros (Xi et al., 2021). Podemos concluir que devido as alterações nas secreções de GnRH e consequentemente de FSH e LH na contra estação reprodutiva, são diversas as alterações na fisiologia reprodutiva dos bodes leiteiros, com redução na síntese e secreção da testosterona que está fortemente ligada a produção de espermatozoides, além de outras regulações das células de Leydig e Sertoli.

2.3 Gonadotrofinas exógenas em reprodutores

Dentre as biotecnologias empregadas para tentar reduzir os efeitos da contra estação, algumas alternativas vêm sendo estudadas, como a utilização de eCG, hCG, melatonina e controle de luz. Assim a utilização de gonadotrofinas exógenas como a gonadotrofina coriônica equina (eCG; Beracochea et al., 2018) e a humana (hCG; Samir et al., 2015) são opções viáveis para induzir efeitos estimulatório semelhantes ao FSH e LH, respectivamente. A utilização de gonadotrofinas tem como principal benefício o rápido efeito e absorção em relação a outras técnicas também empregadas, como a luz artificial (Delgadillo et al. 2004) ou implantes de melatonina (Chemineau et al. 1992), que como já observado em fêmeas os resultados demoram longos períodos para serem observados.

Após aplicações seriadas de altas doses de eCG (500 e 800 IU) foram observados níveis elevados de testosterona sérica em bodes durante a contra estação reprodutiva, também houve aumento da motilidade espermática e motilidade progressiva e melhoria na proporção de espermatozoides com membrana plasmática funcional (Beracochea et al., 2018). Porém este efeito nas qualidades seminais só foi observado até a terceira dose, quando os dois grupos se tornaram semelhantes até o final do estudo. Apenas a testosterona manteve-se elevada durante todas as administrações. Outro ponto observado neste estudo foi a elevação gradual dos níveis de anticorpos contra a eCG, que é algo já bastante discutido na literatura com trabalhos realizados em fêmeas (Roy et al., 1999; Forcada et al., 2011). Um estudo seguinte deste mesmo grupo, observou que na estação reprodutiva seguinte não houve efeito residual ou prejudicial em relação as concentrações de testosterona ou qualidade seminal do sêmen fresco ou pós descongelamento, para os animais que receberam a estimulação gonadal na contra estação anterior, mesmo apresentando níveis elevados de anticorpos três meses após o final do experimento (Beracochea et al., 2019).

A hCG ao mesmo tempo é uma ótima opção, considerando que a sua ação principal é altamente similar ao LH, com alta afinidade por receptores de LH (Stenman et al., 2006), que na fisiologia do macho, estará estimulando

diretamente as células de Leydig, responsáveis pela síntese de testosterona no testículo, porém da mesma forma que com a eCG, existem poucos trabalhos com a utilização destas gonadotrofinas em caprinos, ainda não sendo elucidado todos os benefícios ou não da utilização desta gonadotrofina. Estudos observaram que a hCG induz um aumento nas concentrações de testosterona em bodes (Abdelrahman, et al., 2019), carneiros (Falvo et al., 1975), touros (Sundby & Farahat, 1978) e cães (Sundby & Ulstein, 1981). Também foi observado um aumento na perfusão sanguínea testicular em comparação com as respostas ao tratamento com GnRH em uma raça miniatura de caprinos (Shiba) (Samir et al., 2015). Em peixes, o hCG mostrou-se uma boa opção para a espermiação, melhorando o tamanho das células e a motilidade e concentração espermática, e ainda levando à redução de células com baixa função mitocondrial (Asturiano et al., 2006). A terapia com hCG também é benéfica para homens com oligozoospermia hipogonádica ou oligozoospermia grave, melhorando a concentração e contagem de espermatozoides após três meses de terapia, com doses semanais (250 µg) (Andrabi et al., 2022).

Como mencionado anteriormente, as células de Leydig respondem aos estímulos de LH, assim como a hCG é capaz de se ligar aos receptores de LH (LHR) (Stenman et al., 2006), produzindo um efeito similar ao LH endógeno, estimulando a síntese de testosterona e aumento da captação de colesterol para dentro das mitocôndrias, principalmente regulando a enzima StAR (Proteína de regulação rápida da esteroidogênese) que regula a captação de colesterol pelas mitocôndrias e subsequente transformação em testosterona (Stocco et al., 1997).

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CAPÍTULO 2 – Artigo elaborado conforme as normas da revista Animal Reproduction Science

Does the use of exogenous hCG in male goats during the non-breeding season induce improvements in testosterone levels and fresh and post-thawing semen?

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Abstract

The study aims to investigate if the use of repeated administration of hCG during the non-breading season improves testosterone concentrations, scrotal/testicular parameters, and sperm quality in male goats. Twenty male goats are divided into two groups (G-Control, n = 10; and G-hCG, n = 10) to receive four administrations (im) of 1 mL of saline or 300 IU of hCG, respectively, at 14-day intervals (D0, D14, D28, and D42). On D-7, D0, D14, D28, D42, and D56 semen samples were collected to be evaluated before and after cryopreservation. Biometric parameters were evaluated the day before the semen collection. Blood samples were obtained on D1, D7, D15, D21, D29, D35, D43, and D49 to hormonal dosage. Serum testosterone concentrations were higher ($P = 0.0005$) in G-hCG (9.6 ± 0.6 ng/mL) compared with G-Control (6.3 ± 0.6 ng/mL). There were no treatment effects ($P > 0.05$) for scrotal/testicular biometry, fresh semen parameters as well as post-thawing semen kinetic characteristics. High mitochondrial membrane potential (MMP) cells were observed ($P < 0.05$) on the last day of evaluation (D56) for G-hCG animals (49.0 ± 6.6 %) compared to G-Control ones (26.5 ± 7.4 %). Also, capacitation status was increased ($P = 0.03$) in the G-hCG (48.3 ± 6.1 %) male goats in comparison to G-Control (33.5 ± 6.5 %). In conclusion, four administrations of 300 IU of hCG at 14-day intervals during the non-breeding season are effective in improving the serum testosterone concentrations and promoting higher sperm capacitation status and live sperm with high mitochondrial membrane potential in male goats.

Keywords: human Chorionic Gonadotropin, mitochondrial membrane potential, plasma membrane integrity, semen cryopreservation, sperm kinetics, sperm capacitation status.

Highlights

hCG treatment is efficient in improving the testosterone concentration in male goats during non-breeding season.

After cryopreservation hCG animals have more live sperm with high MMP potential.

High percentage on capacitated sperm after hCG treatment.

1. Introduction

Most goat breeds have a seasonal reproductive pattern, which includes variations in the concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, semen characteristics, and seminal plasma composition throughout the year (Chemineau & Delgadillo, 1993; Barkawi et al., 2006). The greater the melatonin synthesis, the greater the neuroendocrine stimulus of gonadotropin release hormone (GnRH) release and, consequently, the greater the concentrations of LH and FSH, culminating in periods of greater fertility (Srinivasan et al., 2009). With low levels of LH in the non-reproductive season, there is a decrease in testosterone synthesis, and consequently reduced testicular biometry, libido, and sperm production (Roca et al., 1991), since sperm production is dependent on testosterone concentration in the testis (Noakes et al., 2001).

Reproductive biotechnologies have been studied to try to circumvent the negative effects of the non-reproductive season. Among the exogenous gonadotropins, human chorionic gonadotropin (hCG) has been identified as a good alternative, because it has activity similar to LH in the testis binding to LH receptors (Huhtaniemi et al., 1978). The hCG is used mainly as an ovulation inducer and for its potent luteotropic activity in females (Fonseca et al., 2021), however, there is a lack of knowledge of all their effects on gonadal stimulation in males. Since the β-subunits of hCG have approximately 80% of homology with LH (Stenman et al., 2006), and Leydig cells have LH receptors, that after binding, stimulate intracellular signaling cascades (Zirkin & Papadopoulos, 2018), it allows for an excellent hypothesis of action and positive effects in males. The long plasma half-life of hCG, with considerable levels for up to 66 hours and in fast and slow phases (Schimitt et al., 1996), can provide both rapid and prolonged effects in stimulating

testosterone synthesis. Leydig cells are responsible for the conversion of cholesterol into testosterone through acute and chronic peaks of LH, with the activation and expression of the transporter protein StAR (steroidogenic acute regulatory protein), which regulates the uptake of cholesterol by the mitochondria and subsequent transformation into testosterone (Stocoo et al., 1997).

The hCG has been shown to induce an increase in testosterone concentrations in male goats (Abdelrahman, et al., 2019), rams (Falvo et al., 1975), bulls (Sundby & Farahat, 1978), and dogs (Sundby & Ulstein, 1981). It also led to an increase in testicular blood perfusion compared to responses to GnRH treatment in Shiba buck (Samir et al., 2015). In fish, the hCG was shown to be a good option for spermiation, improving the size of the cells and sperm motility and concentration, and even leading to a reduction of cells with low mitochondrial function (Asturiano et al., 2006). The hCG therapy also is beneficial to men with hypogonadic oligozoospermia or severe oligozoospermia, improving the sperm concentration and count after three months of therapy (Andrabi et al., 2022).

Given the above, we hypothesize that administrations of hCG in male goats (Capra hircus, Linnaeus, 1758) during the non-reproductive season induce an increase in serum testosterone concentrations and, consequently, positive effects on the characteristic's indicative of seminal and scrotal/testicular quality. The objective of this study is to evaluate the effects of four repeated doses of 300 IU of hCG, at 14-day intervals, in adult male goats during the non-reproductive season on scrotal/testicle biometric characteristics, serum testosterone concentrations and characteristics of the fresh and post-thawing semen.

2. Material and methods

All experimental procedures were approved by the Ethics Committee on the Use of Animals (CEUA nº 5755150721), from Embrapa Dairy Cattle. The study was conducted during the non-breeding season (August to October, in the spring season), in the Embrapa Dairy Cattle Experimental Farm, located in Coronel Pacheco, Minas Gerais, Brazil (21°38′9″ S, 43°19′9″ W). In the southeast region of Brazil, goats have typically seasonal behavior (Dias et al., 2017). A total of healthy 20 male goats (mixed dairy breeds) with 2.2 ± 0.4 years old, 56.8 ± 12.1 kg of body weight (BW), and 3.0 ± 0.1 of body condition score (BCS, 1-5 scale; Villaquiran et al., 2004) were used. The reproductive health of all experimental male goats was confirmed two months before the start of the study. The animals remained housed in a confined system, fed with corn silage, and supplemented with feed (mix with soybean meal and corn bran), access to fresh water, and mineral salt *ad libitum*. Initially, all males were conditioned regarding human contact and trained to collect semen, using the artificial vagina method.

2.1 Treatments

The males were homogeneously divided according to BW (56.9 ± 10.3 and 56.7 ± 13.9 kg), BCS (3.0 ± 0.1 and 3.0 ± 0.2), and age (2.2 ± 0.4 and 2.1 ± 0.3 years old) into two groups, respectively, to receive or not treatment with hCG: hCG group (G-hCG, n = 10) received four i.m. administrations of 300 IU of hCG (Fertcor®, CEVA, Campinas - SP, Brazil) every 14 days, corresponding to the days (D0 - random day, D14, D28 and D42); and Control Group (G-Control, n = 10), received four administrations (im) of 1 mL of saline solution on the same days of G-hCG treatment. The experiment was conducted in blocks due to the work capacity of the team in the laboratory. For this, the animals were divided into four blocks, with the same number of animals, and similar age, BW, and BCS

of the animals. The experimental activities for each block started one day after the other, therefore all activities were carried out in the same experimental scheme (Figure 1).

2.2 Blood sample collection and hormone measurements

Blood samples were obtained in the morning (from 7:30 to 8:00), on the following days: one week before the starting treatments (D-7); immediately before the first dose of hCG or saline solution (D0 – first dose); D1; D7; D14 (second dose); D15; D21; D28 (third dose); D29; D35; D42 (fourth dose); D43; D49; and D56. Blood samples were collected by puncturing the jugular vein with a needle and vacuum tubes. The blood sample was centrifuged for 20 minutes at 1500 g, and the serum was stored in a freezer at -20ºC until hormone measurement. Serum testosterone concentrations were measured in a single assay, by the solid phase radioimmunoassay (RIA) technique, following the manufacturer's recommendations of the commercial kit (Beckman Coulter, Immunotech, Prague, CzechRepublic). The intra-assay coefficient of variation was 8%.

2.3 Scrotal/testicular biometric parameters

Biometric assessments were conducted on six days (D-8, D-1, D13, D27, D41 and D55). Scrotal circumferences (SC) were obtained with the aid of a scrotal measuring tape, in cm, on the largest portion of the scrotum. Dorsoventral and mediolateral measurements of the right and left testicles were determined with the aid of a digital caliper, in mm. Testicular volume (V) was calculated, in mm^3, using the cylinder volume formula described by Fields et al. (1979): $V = 2\ \{(R^2) \times \pi \times TL\}$, where R = radius (diameter/2), TL = testicular length and $\pi = 3.14$.

2.4 Semen sample collection and fresh semen analysis

Seminal samples were collected by artificial vagina method in the morning for several days (D-7, D0, D14, D28, D42, and D56), with the help of a female estrogenized by the administration of estradiol benzoate (Sincrodiol®, OuroFino, Cravinhos – SP, Brazil) as a mannequin. Semen samples were immediately evaluated regarding volume, color, odor, and appearance, and taken to the laboratory to continue the seminal evaluations.

Sperm mass movement was then assessed by placing a drop of fresh semen (~ 10μL) on a slide preheated to 37ºC, without a coverslip, and placed under a microscope with 10 to 20x objectives. Following what is recommended by CBRA (2013), the interpretation continues using a scale of 0 – 5, with 0 being a total absence of movement and 5 being the maximum value given to mass movement. Then, after adding commercial diluent (Optidux®, Reprodux, Itapira – SP, Brazil) in a 1:1 ratio to the volume of the ejaculate, sperm motility, in percentage, was assessed with a droplet of pre-diluted semen between the slide and coverslip, at a magnification of 10 to 40x, with a plate heated to 37ºC.

Sperm vigor, a subjective assessment that represents the intensity of sperm movement with progressive motility was evaluated, and the result was expressed as a score from 0 to 5, with 0 being the absence of progressive movements with weak displacement, and 5 being the maximum vigor of sperm.

Sperm concentration was obtained under the Neubauer method, a fraction of fresh semen was diluted at a rate of 1:400, wherein 5 μL aliquots of fresh sperm were transferred to 3 mL conical tubes pre-filed with 1995 μL of distilled water. The diluted sample was then evaluated under phase contrast microscopy (x400) in an improved Neubauer hemocytometer chamber and the cell count was due according to WHO (2021).

Sperm cells were evaluated for morphological characteristics of the acrosome, head, midpiece insertion, midpiece, and tail. The evaluation was conducted under a contrast-enhanced phase microscope, after fixation with formaldehyde saline solution, and

following the standard classification for Sperm morphology recommended by Blom (1973). Data were presented in the percentage of the normal morphology spermatozoa.

2.5 Cryopreservation of semen

All semen samples collected were diluted with a ready-to-use commercial product (Optidux®, Reprodux, Itapira – SP, Brazil). Aliquots were stored at a concentration of 480 x 10^6 motile sperm/mL in 0.25 mL straws (Minitube, Tiefenbach, Germany), which were subjected to a slow cooling process for 6 hours in a Styrofoam box at 5ºC. Each animal had an individual Styrofoam box to keep the controlled inner temperature. After that, the straws were placed upon racks suspended 6 cm above the surface of liquid nitrogen for 15 minutes. Then they were immersed directly in nitrogen to reach -196°C, and the samples were racked and stored in a liquid nitrogen cylinder for at least 30 days until thawed to carry out evaluations.

2.6 Post-thawing semen assessment

The cryopreserved semen samples were subjected to thawing, a step that involves removing one straw from the liquid nitrogen cylinder and immediately immersing it in a water bath at 37ºC for 30 seconds. After drying the straw externally, its contents were transferred to a preheated 2 mL plastic tube and kept in a water bath at 37°C. The samples were then subjected to evaluation using the computer-assisted objective semen analysis (CASA) system using the SCA system (Sperm Class Analyzer Microptic, Nikon Eclipse Ci – Tokyo, Japan), with the software configured for goat semen. The assessments to be carried out are described below.

2.6.1 Sperm kinetics characteristics

Motility parameters were analyzed by the CASA system with configured a default setting size of the sperm head detectable between 18 and 60 μm^2. Five images were captured at 100x magnification. Assessments were carried out on a 24 × 24 mm coverslip with a 10 µL drop of each sample. Sperm was considered immobile when the curvilinear velocity (VCL) was below 10 µm/s; slow when between 10 and 45 µm/s; medium when between 45 and 75 µm/s; and fast when above 75 µm/s. Sperm cells showing linearity (STR) above 80% were characterized as progressive motile sperm. The following kinetic characteristics were determined: total motile spermatozoa (%), progressive motile spermatozoa (%), fast spermatozoa (%), medium speed spermatozoa (%), slow speed spermatozoa (%), average travel speed (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), lateral amplitude head displacement (ALH, µm/s), beat/cross rate (BCF, Hz), straightness (STR, %), linearity (LIN, %) and WOB (average value of the ratio between VAP and VCL, %).

2.6.2 Hypoosmotic test

A hypoosmotic test was performed to evaluate the integrity of the sperm membrane. A drop of 30 µL thawed semen was used and diluted in 1 mL of the hypoosmotic solution of 100 mOsm/kg of MilliQ water (Bucak et al., 2009). This sample was incubated in a water bath at 37 ºC for 20 min. A drop (10 µL) of this sample was placed on a slide and covered with a coverslip for evaluation under an optical microscope at 400x magnification. The assessment was carried out following the standards stipulated by CBRA (2013). A total of 200 sperm cells were evaluated for the presence of a coiled tail (cells with an intact plasma membrane) or not, and the result was expressed as a

percentage of sperm with an intact plasma membrane in the hypoosmotic test. The result obtained after subtracting the number of sperm with a bent tail, observed by the sperm morphology test, following the formula: HOS (%) = (% of changes in the tail region in the HOS test) – (% of changes in the sperm tail region before the HOS test).

2.6.3 Epifluorescence microscopy assessment

Analyses of plasma membrane integrity, mitochondrial activity, and sperm capacitation status were performed at an epifluorescence microscope with a protected filter for optimized reading (Nikon Eclipse Ci- Nikon Corporation, Japan). For each staining protocol, samples were distributed on a glass slide overlaid with a coverslip and observed under the designated magnification for each analysis. At least 200 sperm cells were assessed per semen sample.

Plasma membrane integrity

The plasma membrane integrity was assessed by the association of two fluorescent probes: acridine orange [10,000x] and propidium iodide (0.5 mg/mL). Acridine orange is a plasma membrane permeable probe selective to nucleic acids that emit green fluorescence. Propidium iodide is a non-plasma membrane permeable probe that binds to nucleic acids and subsequently emits red fluorescence. The analysis was performed on a microscope under epifluorescence illumination (100x) equipped with an appropriate filter set (465–495 nm excitation and 515–555 nm emission). At least 300 sperm per slide were analyzed. The cells were classified according to their color: cells stained in green were considered to have an intact membrane and those stained in red were considered to have membrane damage.

Mitochondrial activity

The mitochondrial activity was assessed utilizing a working solution of 4 mL of buffered saline and 10 µL of MitoTracker (MitoTrackerGreen®, Cell Signaling Technology, Danvers – MA, USA) previously prepared (final concentration of 20 µM). The semen sample (10 µL) was diluted in MitoTracker working solution (90 µL), incubated for 30 min at 37 °C inside a plastic tube (1.5 mL), and then evaluation was immediately conducted on epifluorescence microscopy. Filter sets: 490 nm excitation and 512 nm emission. MitoTracker Green, labels mitochondria of live spermatozoa only (Druart et al., 2009). The classification was done visually through the microscope evaluating the intensity of the cells in high, low, or inactive mitochondrial membrane potential (MMP), and expressed in percentage.

Sperm capacitation status

The state of sperm capacitation was assessed by the chlortetracycline (CTC) test (Cormier et al., 1997; Alfradique et al., 2018). A 0.75 mM CTC work solution (pH 7.8) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM 1-cysteine. The sperm sample (5 µL) was mixed with an equal volume of CTC solution, and after a few seconds, 2 µL of 2% paraformaldehyde was added. A blue-violet 2 A (BV-2 A) filter with 400–440 nm excitation and 470 nm emission fluorescence was used. Classification according to the criteria suggested by Cormier et al. (1997): uniform bright fluorescence over the whole head (non-capacitated), fluorescence-free band in the post-acrosomal region (capacitated), and dull fluorescence over the whole head with a thin and bright band of fluorescence along the equatorial region (acrosome reacted cells).

2.6.4 Sperm morphology

Sperm morphology on thawed semen was under the same methodology as the fresh semen described before.

2.7 Statistical analysis

For statistical analyses, the SAS® software (Statistical analysis software) was used. Tests of normality of residuals and homoscedasticity were conducted. The analysis of the data was conducted with the mixed model procedure (PROC MIXED) and a repeated statement was used for evaluation, with treatment, and interaction of treatment and time as the main effects in the model, and with random effects block and animal age. The D-7 data (Table 1) were used as the covariate. The significance value adopted was $P < 0.05$, and tendencies when $0.05 < P \leq 0.1$. Data were expressed as LSmeans ± SEM.

3. Results

3.1 Testosterone concentrations

The serum testosterone concentration was greater ($P = 0.0005$) in G-hCG male goats (9.6 ± 0.6 ng/mL) compared to G-Control ones (6.3 ± 0.6 ng/mL) (Figure 2). There was also an interaction between treatment and time effects ($P < 0.0001$); greater concentrations were observed in G-hCG male goats on D1, D15, and D29 (12.2 ± 2.1, 18.1 ± 2.1, and 12.7 ± 2.1 ng/mL) compared to those from G-Control (5.2 ± 2.1, 5.3 ± 2.1, and 5.0 ± 2.2 ng/mL), respectively.

3.2 Scrotal/testicular biometric parameters

The scrotal/testicular biometric parameters and fresh semen analysis are presented in the Figure 3.

An interaction effect between treatment and time, was observed for scrotal circumference, when the G-Control animals (25.7 ± 0.7 cm) had greater values ($P = 0.04$) on D13 compared to G-hCG ones (24.7 ± 0.7 cm).

Testicular volume did not differ between treatments (G-Control: 490.6 ± 22.4 mm^3 vs. G-hCG: 448.2 ± 21.5 mm^3) Furthermore, an interaction effect between treatment and time was also observed in the testicular volume, when G-Control males, tended (P = 0.08) to be greater than G-hCG on D41 (529.8 ± 27.5 mm^3 vs. 462.3 ± 27.0 mm^3) (Figure 3C).

3.3 Fresh semen characteristics

For semen volume, there was an interaction between treatment and time (P = 0.03), when a greater seminal volume was observed on D0 to G-Control male goats (0.9 ± 0.1 mL) compared to G-hCG male goats (0.5 ± 0.1 mL) (Figure 3D).

Motility percentage tended (P = 0.05) to be higher for G-Control male goats than G-hCG (G-Control: 85.2 ± 1.9 % vs G-hCG: 82.0 ± 1.8 %, Figure 3E).

For semen vigor, an interaction effect was observed between treatment and time, when on D14, G-Control male goats (4.4 ± 0.2) had more vigor than G-hCG (3.6 ± 0.2) (Figure 3G).

3.4 Kinetics characteristics of post-thawing semen

The results for kinetics characteristics of post-thawing were presented in the Figure 4. No differences were observed for all the kinetics characteristics of post-thawing semen.

3.5 Other characteristics of pos-thawing semen

Capacitated sperm percentage was observed a treatment effect when G-hCG (48.3 ± 6.1 %) had higher value than G-Control (33.5 ± 6.5 %), Figure 4A. Also, and interaction effect was observed when, on D56 G-hCG (36.5 ± 8.8) male goats had more percentage than G-Control (11.5 ± 9.0 %).

No difference was observed for non-capacitated sperm percentage between treatments (G-Control: 40.0 ± 8.0 % vs G-hCG: 40.3 ± 8.3 %), Figure 4B.

For acrosome-reacted cells percentage, an interaction between treatment and time was observed, when on D28 G-Control (44.9 ± 8.2 %) male goats had higher values than G-hCG (22.6 ± 7.4 %) (Figure 5C).

The high MMP percentage had an interaction effect between treatment and time was observed, on D56 G-hCG male goats (48.9 ± 6.6 %) had higher percentage than G-Control (26.5 ± 7.4 %) (Figure 5D).

The low MMP percentage did not differed between treated and untreated bucks (G-Control: 52.9 ± 4.0 vs G-hCG: 51.2 ± 3.7 %) (Figure 5E)

The inactive MMP did not differ between treated (G-hCG: 23.0 ± 4.9 %) and untread bucks (G-Control: 28.5 ± 5.2 %) (Figure 5F).

4. Discussion

In view of our searches in the scientific literature, we consider that this is the first study designed to test the hypothesis that four repeated doses of 300 IU of hCG in adult male goats, at 14-day intervals, during the non-reproductive season induces an increase in serum testosterone concentrations and, consequently, positive effects on the characteristic's indicative of seminal and scrotal/testicular quality. To confirm our hypothesis, an increase of ~ 2.7 ng/mL in the overall mean serum testosterone concentrations was confirmed in G-hCG compared to G-Control. Additionally, higher concentrations in G-hCG were seen one day after the first three doses of hCG compared to G-Control. Additionally, on D56 the percentage of live spermatozoa with high MMP and the percentage of capacitated sperm were higher for treated animals.

In overall the administration of four doses of hCG improved the testosterone concentrations in male goats, and a tendency of interaction from the effects was observed, demonstrating that the time was also a factor, maybe duo to the fact of multiple sperm

collection and the method of collection, also the social contact of the males during the experiment might induced a stimulation of the pituitary gland, and releasing more hormones (Bahadi et al., 2023). Although the frequency of administration used increased testosterone concentrations at specific moments, they did not remain high for the expected time. In a breed of miniature male goats, a single injection of hCG (25 IU/kg BW) was utilized and the testosterone peak observed was on the fourth day, with a subsequent reduction until the seventh day (Samir et al., 2015), being similar to what was observed in our male goats. In agreement with another study that utilized a different gonadotropin stimulation (eCG; Beracochea et al. 2018), our findings maybe walk through the same way, in a production on anti-bodies against the hCG, and maybe duo that we didn't see a high elevation of testosterone after the third administration. It would be interesting to perform studies with more animals, to test variations in the protocol, including possible modifications in the intervals used and, in the doses, and to evaluate the results of the fertility from this improved semen.

Under the conditions used in this study, no effects were observed after the treatment for testicular biometric parameters, fresh semen quality and post-thawing sperm kinetics characteristics. The testicular volume increased along the time, being higher at the end of the experiment, that maybe duo to the multiple semen collections and the social stimulation by the contact with the estrogen female and other males. Similar results were observed from other studies when control animals have higher scrotal circumference or the same values of testicular volume as the treated animals (Samir et al., 2015; Beracochea et al., 2018). The seminal volume was similar most of the time, even though there is not a greater amount of volume for the treated animals, just there is no drop in production as was observed with the control animals, this is something interesting to consider for the non-breeding season.

Since sperm motility is highly correlated with MMP (Agnihotri et al., 2016), the progressive increasement in high MMP live cells from treated animals support the idea of an improvement in this parameter even after the end of treatment, as mitochondria in sperm cells are responsible for ATP production, that is used for the sperm motility (Ruiz-Pesini et al., 2000). When Leydig cells are stimulated by the rise of testosterone, it leads to an indirect stimulation of Sertoli cells by the peritubular cells, resulting on more nutrients incoming for germ cells (Skinner, 1987), this sequence of biological mechanisms was observed in fish after hCG treatments, when thick cells with more width and length were observed (Asturiano et al., 2006). Our hypothesis is that the increase of nutrients resulted by the hCG stimulation of the Leydig cells may be the cause of the increase in the percentage of living cells with mitochondrial potential observed in the on the lasts semen collections of our study, when hCG treatment affects the expression of enzymes (P450-ssc or P450-17α) related to energy production in mitochondrial flagella and germ cells mitochondria (Guais et al., 2004). That way, hCG shows to improve the spermatozoa mitochondria in many ways during his production and maturation until the ejaculation. And as hCG have long-term effects in Leydig cells and the cascade that triggers in testicular cells (Skinner, 1987) providing more nutrition for the cells, this corroborates with our findings for mitochondrial analysis.

In conclusion, hCG is an efficient tool for providing more testosterone concentrations for male goats using 300 UI, and overall providing a greater sperm capacitation status and live sperm with high mitochondrial membrane potential after the gonadotrophic stimulation during the non-breeding season.

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Table legends.

Table 1. Mean ± SEM of variables related to the male goats biometric/semen status one week before (Day -7) the start of the study used as covariate for statistical analysis, from G-Control and G-hCG animals.

Figure legends.

Figure 1. Experimental study design that tests the use of four doses of hCG (300 IU, im) with 14 days of interval in male bucks during the non-breeding season. On days 0, 14, 28 and 42, either 300 UI of hCG or 1 mL saline solution were administrated (im) in G-hCG and G-Control, respectively. Semen collections were performed on days -7, 0, 14, 28, 42 and 56 for fresh semen analysis, cryopreservation and posterior post-thawing semen analysis. One day before each semen collection, biometric parameters were measured. Also, blood samples were obtained for serum testosterone evaluations.

Figure 2. LSMeans (± SEM) of serum testosterone concentrations of samples collected during the non-breeding season from male goats that were treated (white triangle) or not treated (black circle) with hCG.

Figure 3. LSMeans (± SEM) of biometrics and fresh semen characteristics from sperm samples collected during the non-breeding season from male goats treated (white triangle) or not treated (black circle) with hCG.

Figure 4. LSMeans (± SEM) of pos-thawing sperm kinetics characteristics from sperm samples collected during the non-breeding season from male goats treated (white triangle) or not treated (black circle) with hCG.

Figure 5. LSMeans (± SEM) of capacitation status, mitochondrial membrane potential, hypoosmotic test, plasma membrane integrity and morphology from post-thawing sperm samples collected during the non-breeding season from male goats treated (white triangle) or not treated (black circle) with hCG.

*. Indicates differences between groups in specific days.

† Indicates tendency between groups in specific days.

Figure 1.

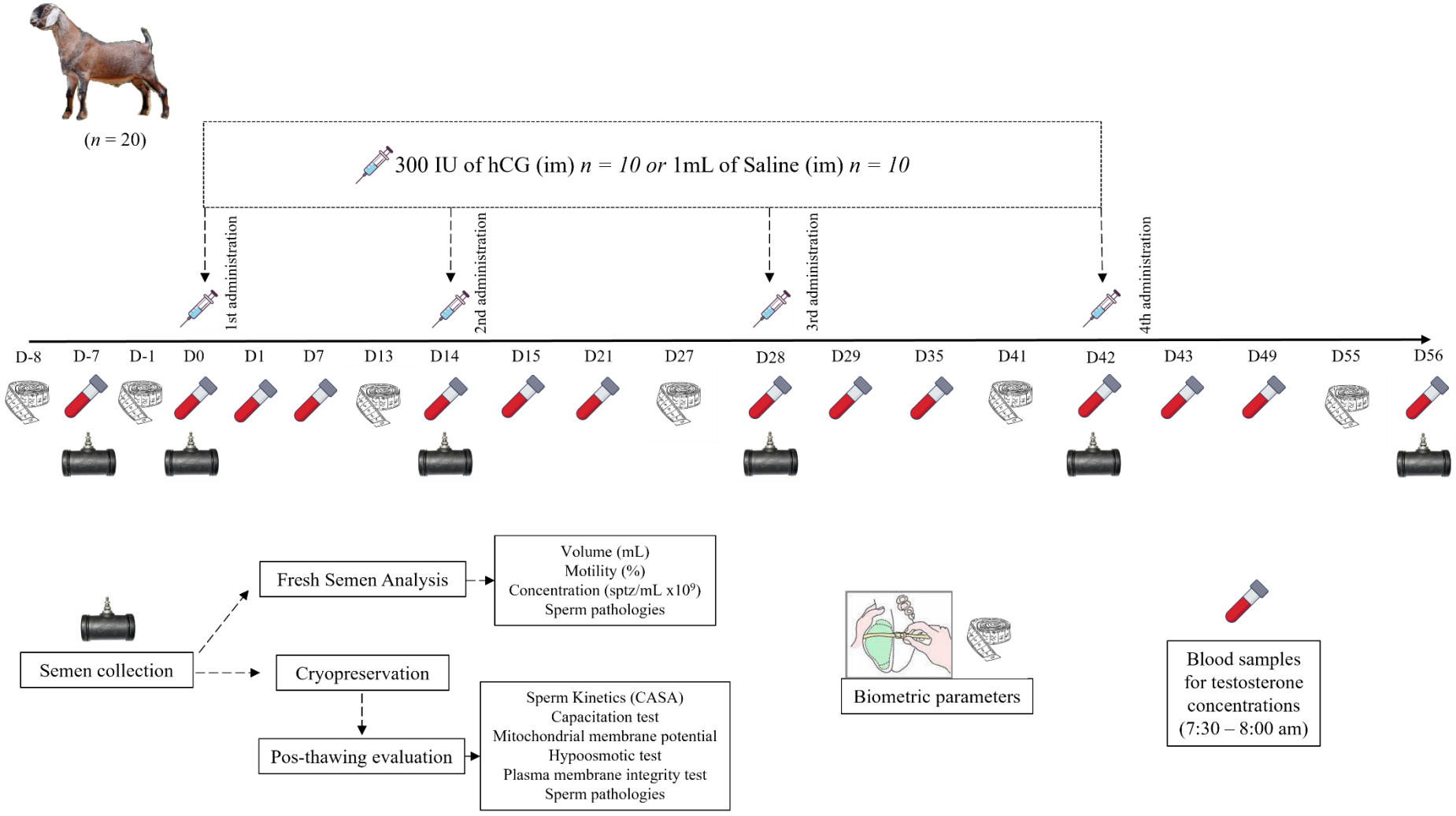

(n = 20)
300 IU of hCG (im) n = 10 or 1mL of Saline (im) n = 10
1st administration
2nd administration
3rd administration
4th administration
D-8
D-7
D-1
D0
D1
D7
D13
D14
D15
D21
D27
D28
D29
D35
D41
D42
D43
D49
D55
D56
Semen collection
Fresh Semen Analysis
Volume (mL)
Motility (%)
Concentration (sptz/mL x10⁹)
Sperm pathologies
Cryopreservation
Pos-thawing evaluation
Sperm Kinetics (CASA)
Capacitation test
Mitochondrial membrane potential
Hypoosmotic test
Plasma membrane integrity test
Sperm pathologies
Biometric parameters
Blood samples for testosterone concentrations (7:30 – 8:00 am)

Figure 2.

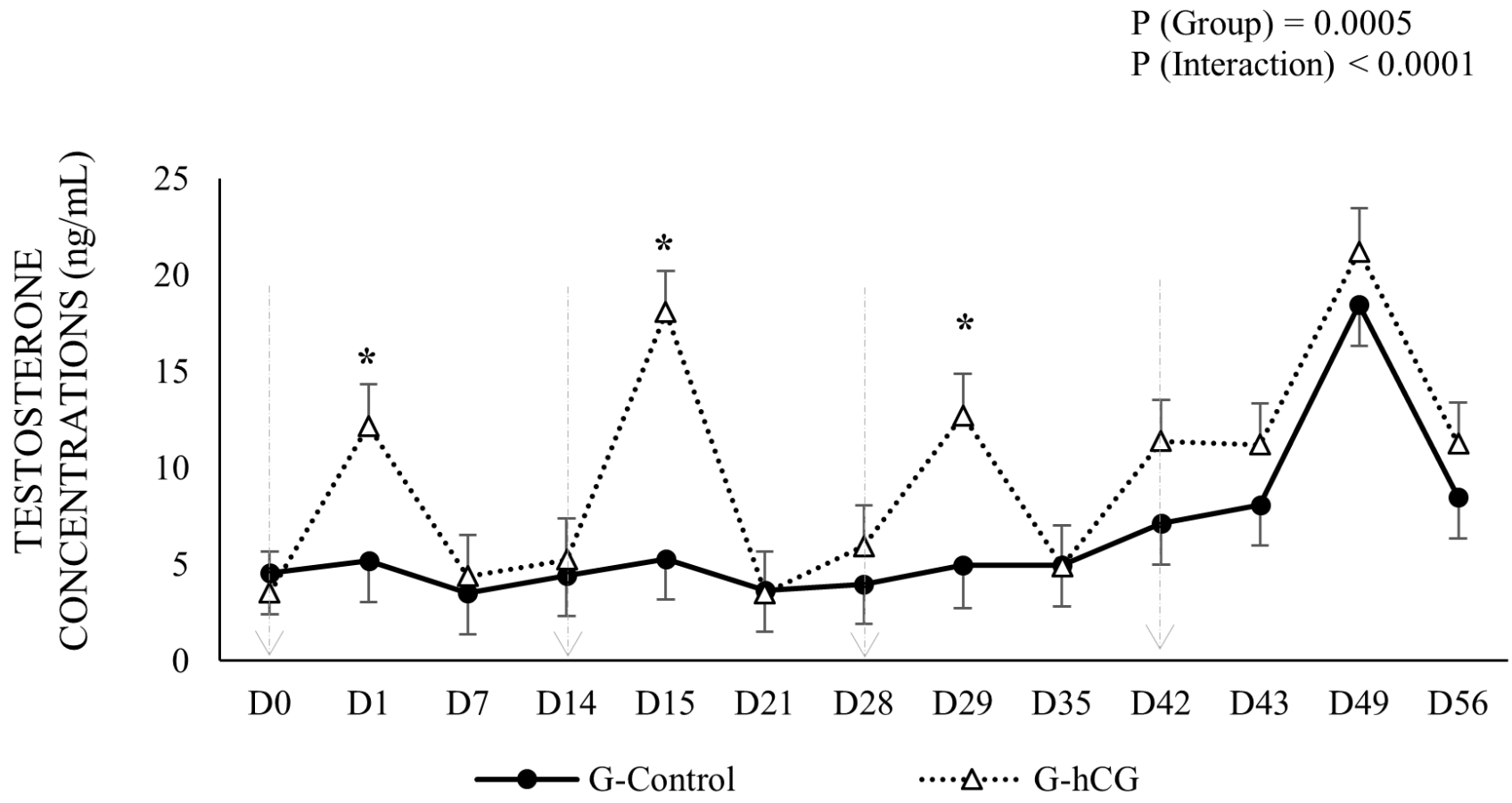

P (Group) = 0.0005
P (Interaction) < 0.0001
TESTOSTERONE CONCENTRATIONS (ng/mL)
25
20
15
10
5
0
*
*
*
D0
D1
D7
D14
D15
D21
D28
D29
D35
D42
D43
D49
D56
G-Control
G-hCG

Figure 3.

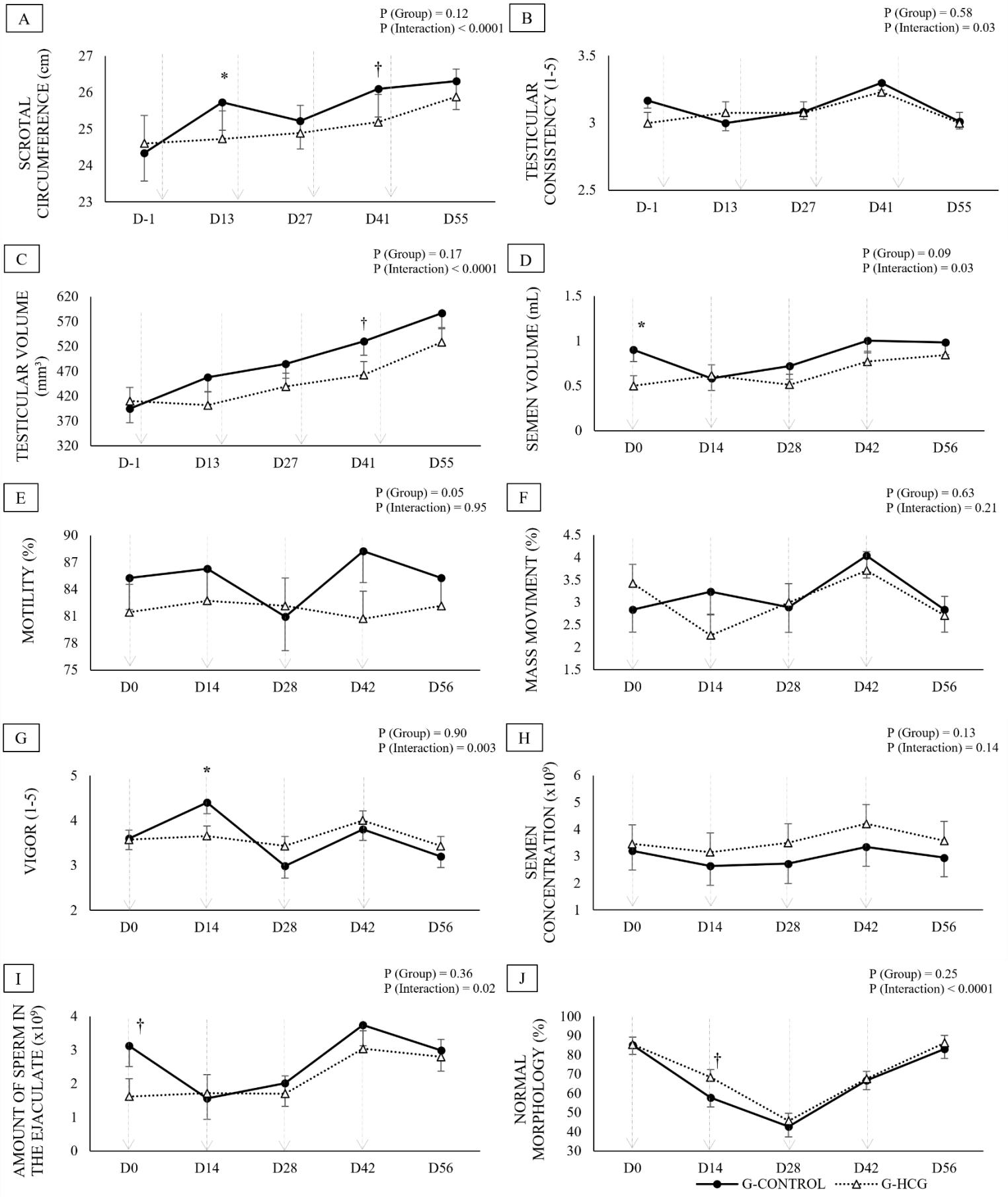

A
P (Group) = 0.12
P (Interaction) < 0.0001
SCROTAL CIRCUMFERENCE (cm)
27
26
25
24
23
D-1
D13
D27
D41
D55
B
P (Group) = 0.58
P (Interaction) = 0.03
TESTICULAR CONSISTENCY (1-5)
3.5
3
2.5
C
P (Group) = 0.17
P (Interaction) < 0.0001
TESTICULAR VOLUME (mm³)
620
570
520
470
420
370
320
D
P (Group) = 0.09
P (Interaction) = 0.03
SEMEN VOLUME (mL)
1.5
1
0.5
0
D0
D14
D28
D42
D56
E
P (Group) = 0.05
P (Interaction) = 0.95
MOTILITY (%)
90
87
84
81
78
75
F
P (Group) = 0.63
P (Interaction) = 0.21
MASS MOVIMENT (%)
4.5
4
3.5
3
2.5
2
1.5
G
P (Group) = 0.90
P (Interaction) = 0.003
VIGOR (1-5)
5
4
3
2
H
P (Group) = 0.13
P (Interaction) = 0.14
SEMEN CONCENTRATION (x10⁹)
6
5
4
3
2
1
I
P (Group) = 0.36
P (Interaction) = 0.02
AMOUNT OF SPERM IN THE EJACULATE (x10⁹)
4
3
2
1
0
J
P (Group) = 0.25
P (Interaction) < 0.0001
NORMAL MORPHOLOGY (%)
100
90
80
70
60
50
40
30
G-CONTROL
G-HCG

Figure 4.

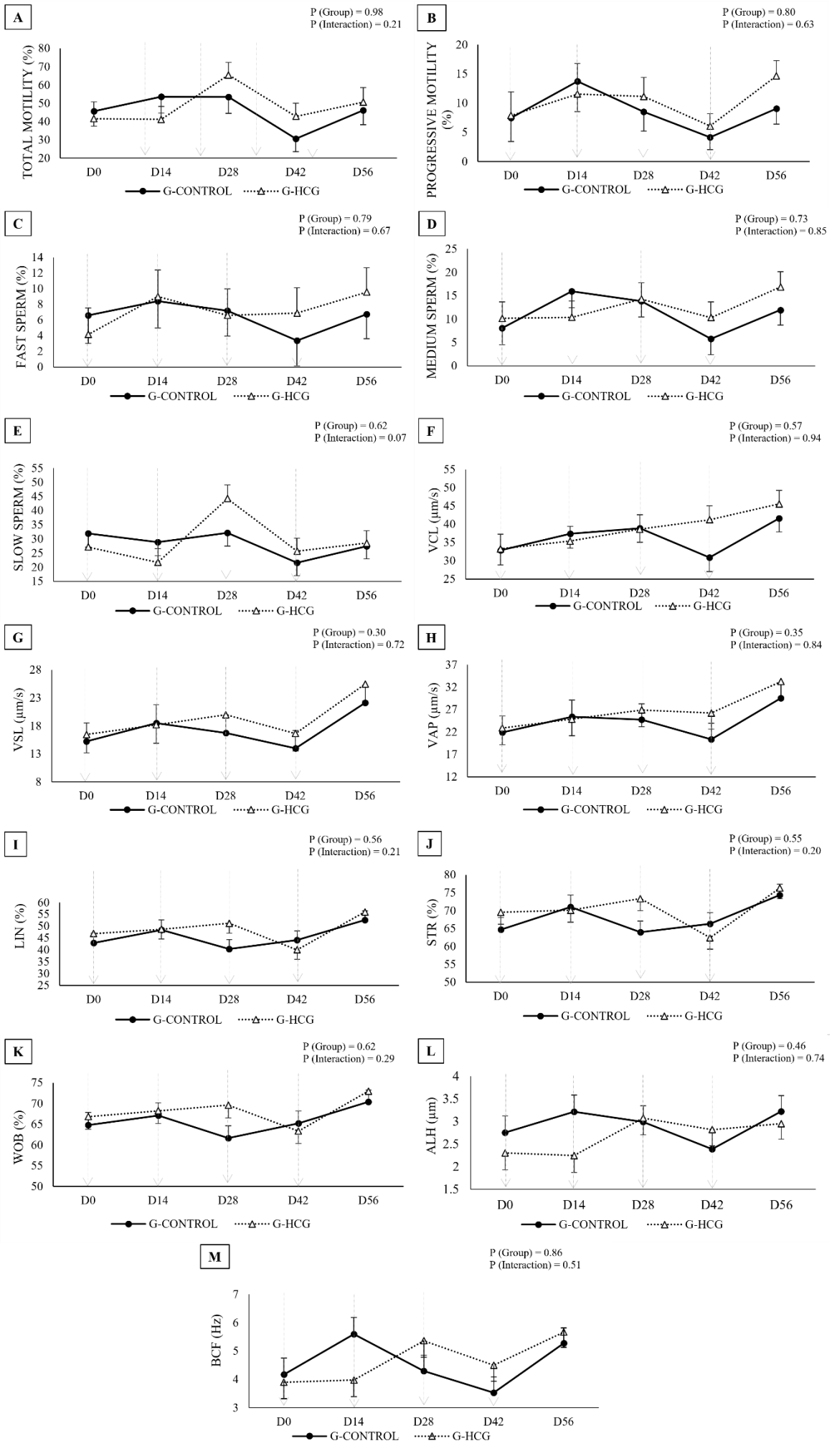

A
P (Group) = 0.98
P (Interaction) = 0.21
TOTAL MOTILITY (%)
B
P (Group) = 0.80
P (Interaction) = 0.63
PROGRESSIVE MOTILITY (%)
C
P (Group) = 0.79
P (Interaction) = 0.67
FAST SPERM (%)
D
P (Group) = 0.73
P (Interaction) = 0.85
MEDIUM SPERM (%)
E
P (Group) = 0.62
P (Interaction) = 0.07
SLOW SPERM (%)
F
P (Group) = 0.57
P (Interaction) = 0.94
VCL (µm/s)
G
P (Group) = 0.30
P (Interaction) = 0.72
VSL (µm/s)
H
P (Group) = 0.35
P (Interaction) = 0.84
VAP (µm/s)
I
P (Group) = 0.56
P (Interaction) = 0.21
LIN (%)
J
P (Group) = 0.55
P (Interaction) = 0.20
STR (%)
K
P (Group) = 0.62
P (Interaction) = 0.29
WOB (%)
L
P (Group) = 0.46
P (Interaction) = 0.74
ALH (µm)
M
P (Group) = 0.86
P (Interaction) = 0.51
BCF (Hz)
D0
D14
D28
D42
D56
G-CONTROL
G-HCG

Figure 5.

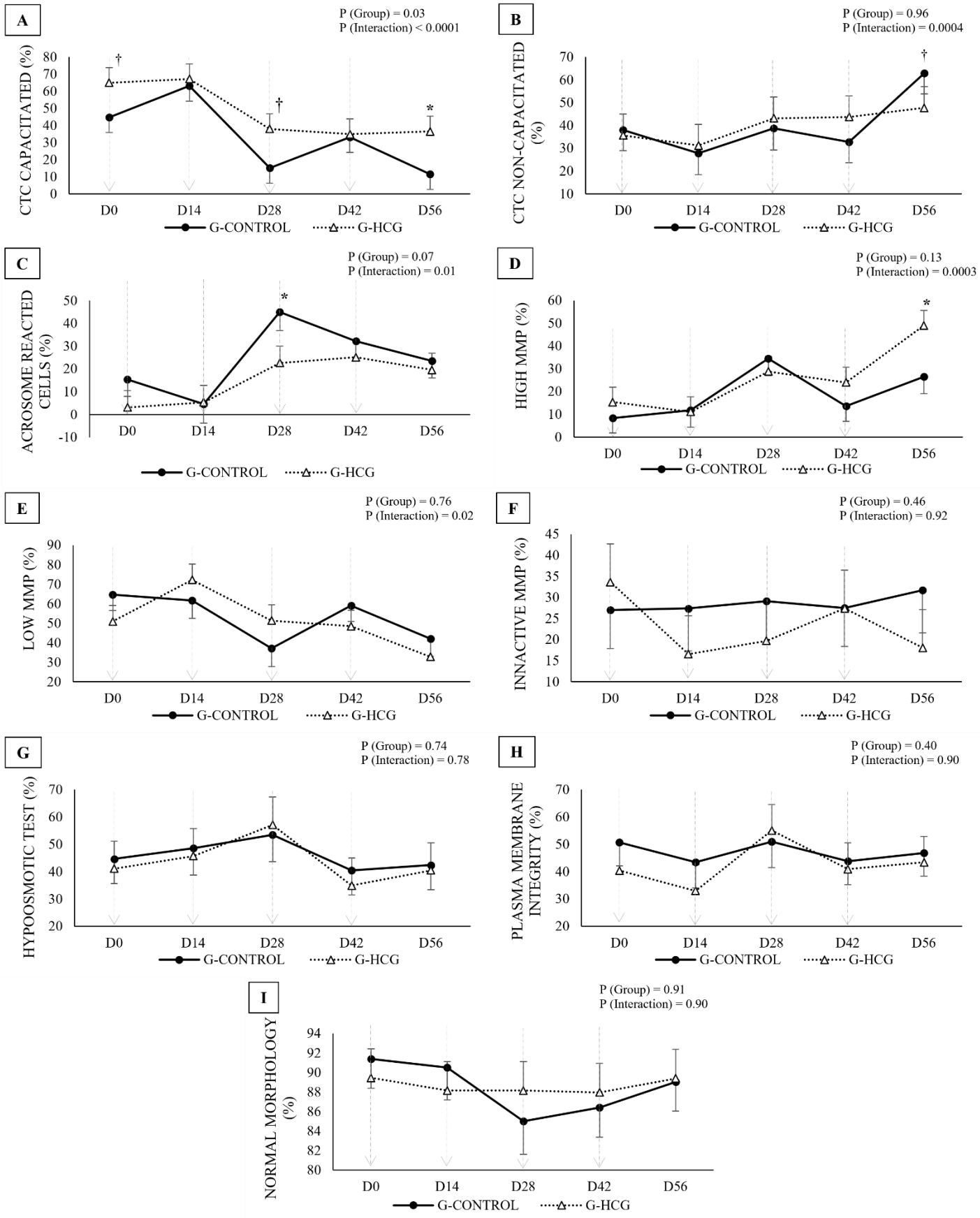

A
P (Group) = 0.03
P (Interaction) < 0.0001
CTC CAPACITATED (%)
D0 D14 D28 D42 D56
G-CONTROL G-HCG
B
P (Group) = 0.96
P (Interaction) = 0.0004
CTC NON-CAPACITATED (%)
C
P (Group) = 0.07
P (Interaction) = 0.01
ACROSOME REACTED CELLS (%)
D
P (Group) = 0.13
P (Interaction) = 0.0003
HIGH MMP (%)
E
P (Group) = 0.76
P (Interaction) = 0.02
LOW MMP (%)
F
P (Group) = 0.46
P (Interaction) = 0.92
INNACTIVE MMP (%)
G
P (Group) = 0.74
P (Interaction) = 0.78
HYPOOSMOTIC TEST (%)
H
P (Group) = 0.40
P (Interaction) = 0.90
PLASMA MEMBRANE INTEGRITY (%)
I
P (Group) = 0.91
P (Interaction) = 0.90
NORMAL MORPHOLOGY (%)

Table 1.

	G-Control	G-hCG
Fresh semen		
Scrotal Circumference (cm)	24.2 ± 0.7	23.3 ± 0.7
Testicular Consistency (1-5)	3.1 ± 0.1	3.1 ± 0.1
Testicular Volume (mm^3)	406.3 ± 44.0	368.2 ± 42.0
Semen Motility (%)	84.0 ± 2.9	87.1 ± 2.4
Semen Volume (mL)	0.8 ± 0.1	0.7 ± 0.1
Sperm Concentration (Sptz/mL $x10^9$)	4.6 ± 0.4	3.7 ± 0.3
Amount of Sperm in Ejaculate ($x10^9$)	3.7 ± 0.7	2.7 ± 0.6
Testosterone Concentration (ng/mL)	2.8 ± 0.5	2.2 ± 0.4
Normal Morphology (%)	86.1 ± 2.9	85.5 ± 3.2
Post-thawing semen		
Total Motility (%)	65.5 ± 1.4	52.0 ± 9.0
Progressive Motility (%)	17.1 ± 5.6	6.0 ± 3.3
Fast Sperm (%)	12.7 ± 5.9	4.5 ± 2.0
Medium Sperm (%)	20.9 ± 1.4	13.5 ± 5.9
Slow Sperm (%)	31.8 ± 4.5	33.9 ± 2.7
Curvilinear Velocity (VCL, mm/S)	48.9 ± 5.5	35.7 ± 4.8
Straight-Line Velocity (VSL, mm/S)	27.2 ± 6.8	13.7 ± 3.1
Average Travel Speed (VAP, mm/S)	36.8 ± 7.1	22.0 ± 3.9
Linearity (LIN, %)	52.6 ± 8.4	36.8 ± 4.5
Straightness (STR, %)	70.3 ± 6.7	60.4 ± 4.1
WOB (Average Value of The Ratio Between VAP and VCL, %)	72.8 ± 6.0	60.2 ± 3.7
Lateral Amplitude Head Displacement (ALH, mm/S)	2.9 ± 0.3	3.0 ± 0.3
Beat/Cross Rate (BCF, Hz)	5.1 ± 0.3	4.1 ± 0.7
CTC Capacitated (%)	60.1 ± 2.1	58.2 ± 9.3
CTC non-capacitated (%)	30.5 ± 2.1	23.3 ± 3.4
Acrossome Reacted Cell (%)	9.3 ± 3.4	18.5 ± 8.9
High MMP (%)	5.9 ± 3.5	5.0 ± 2.9
Low MMP (%)	64.1 ± 5.8	53.2 ± 6.5
Inactive MPP (%)	30.0 ± 7.9	41.7 ± 9.0
Hypoosmotic Test	41.2 ± 6.2	50.2 ± 3.7
Plasma Membrane Integrity (%)	58.7 ± 6.2	49.8 ± 3.8
Normal Morphology (%)	90.4 ± 3.5	85.8 ± 3.8