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SIMONE SOMMERFELD

Embriões de galinha como modelo experimental para testes com fármacos e vírus

Uberlândia

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Dissertação apresentada ao Programa de Pós-graduação em Ciências Veterinárias da Faculdade de Medicina Veterinária da Universidade Federal de Uberlândia como requisito parcial para obtenção do título de mestre em Ciências Veterinárias.

Área de concentração: Saúde animal

Orientador: Prof Dr Antonio Vicente Mundim

Coorientador: Profª Drª Belchiolina Beatriz Fonseca

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A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

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Área de concentração: Saúde Animal

Uberlândia, 30 de agosto de 2021

Banca Examinadora:

_____
Antonio Vicente Mundim – Doutor (FAMEV-UFU)

_____
Marcelo Emílio Beletti – Doutor (ICBIM-UFU)

_____
Murilo Vieira da Silva – Doutor (ICBIM-UFU)

_____
Roselene Ecco – Doutora (EV-UFMG)

Dedico este trabalho aos meus pais e marido pelo estímulo, carinho e compreensão.

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“Ninguém ignora tudo. Ninguém sabe tudo. Todos nós sabemos alguma coisa. Todos nós ignoramos alguma coisa.”

(FREIRE, 2002, p. 69)

RESUMO

Ao longo dos anos o embrião de galinha (EG) tem sido um modelo animal muito usado; no entanto, há uma constante falta de padronização do uso de embriões. Sendo assim, o objetivo deste estudo é verificar se alterações macroscópicas, histológicas, hematológicas, metabólicas e de estresse oxidativo em EG de diferentes idades são peculiares ao modelo, propondo a necessidade de uma padronização tendo como referência um vírus e dois fármacos que causam alterações conhecidas em adultos e outras espécies. Os EG foram incubados a 37ºC e 58% de umidade e virados automaticamente a cada duas horas. Aos 10 dias de incubação embrionária (DIE), EG foram inoculados com *Gammacoronavirus* no líquido alantóide tendo o controle (CN) em paralelo. Para o teste de drogas, foram realizadas inoculações com Filgrastim (FG) (150µg/EG) e dexametasona (DX) (0,08µg/EG) e um grupo CN nas idades de 0, 3, 7, 10 e 12 DIE via membrana da casca (MC) e via membrana corioalantoide (CAM). Os EG foram pesados, a mortalidade e as lesões macroscópicas foram avaliadas. Além disso, foram coletadas amostras para análise bioquímica, hematológica, exames histopatológicos e estresse oxidativo. A resposta dos embriões aos desafios com vírus e fármacos nem sempre ocorre conforme o esperado. Embora as alterações macroscópicas e microscópicas fossem visíveis nos infectados, as contagens de leucócitos e os biomarcadores de inflamação, como a proteína C reativa, não alteraram. É importante observar que algumas drogas podem não resultar efeitos esperados para o EG como constatado no tratamento com FG. Para os embriões tratados com DX, alterações nos parâmetros sanguíneos e biomarcadores parecem ser inerentes ao modelo e são dependentes do estágio de desenvolvimento do EG.

Palavras-chave: membrana corioalantóide, gammacoronavírus, toxicicidade

ABSTRACT

For many years the chicken embryo (CE) has been a widely used animal model, however, there is a constant lack of standardization in the use of these animals. Thus, the objective of this study is to verify if macroscopic, histopathological, blood count, metabolic changes and oxidative stress in CE of different ages are peculiar to the model, proposing the need for standardization based on two drugs and a virus that cause known changes in other animals. The CE were incubated at 37ºC and 58% humidity and turned automatically every two hours. At 10 embryonic incubation days (EID), CE were inoculated with *Gammacoronavirus* in allantoic fluid having the negative control (NC) in parallel. For drug testing, inoculations were performed with Filgrastim (FG) (150µg/CE) and dexamethasone (DX) (0.08µg/EG) and a NC group at the ages of 0, 3, 7, 10 and 12 EID via shell membrane (SM) and chorioallantoic membrane (CAM). CE were weighed and mortality and macroscopic lesions were assessed. In addition, samples were collected for biochemical, hematological, histopathological and oxidative stress analysis. The response of embryos to challenges with viruses and drugs does not always go as expected. Although macroscopic and microscopic changes were visible in those infected, white blood cell counts and inflammation biomarkers such as C-reactive protein did not change. It is important to note that some drugs may not result in expected effects for CE as seen in FG treatment. For embryos treated with DX, changes in blood parameters and biomarkers seem to be inherent in the model and are dependent on the stage of CE development.

Keywords: chorioallantoic membrane, gammacoronavirus, toxicity

SUMÁRIO

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

Ao longo dos anos o embrião de galinha (EG) tem sido um modelo animal muito usado, pois os ovos fertilizados são economicamente mais acessíveis, fáceis de serem adquiridos e estão disponíveis em grande número durante todo o ano. Possui desenvolvimento acelerado quando comparado aos embriões dos mamíferos, sendo equivalente apenas ao do camundongo, durando 21 dias. Sua genética é bem conhecida e, sendo um amniota, o desenvolvimento embrionário se assemelha ao de um mamífero, porém com fisiologia menos complexa devido à ausência de uma placenta e, consequentemente, de eventos que possam ocorrer dentro da mãe [1].

O EG tem sido aplicado para avaliar inúmeros parâmetros como angiogênese, isquemia, desenvolvimento e tratamento de câncer, sistema de entrega de fármacos, toxicidade, entre outros [2–5]. Estudos com medicamentos usando EG como modelo podem produzir resultados similares gerados em camundongos, sendo uma alternativa para testes preliminares aos estudos em mamíferos como forma de economizar tempo, material e animais [6,7]. São muito utilizados para estudos de câncer e metástase por oferecer um ambiente de suporte para o desenvolvimento de tumores com grande quantidade de vasos sanguíneos e angiogênese [8,9]. O EG em desenvolvimento fornece um sistema hospedeiro potencial com diversidade de tipos celulares que são necessários para a replicação bem-sucedida de uma ampla variedade de vírus [10] sendo, desta forma um bom modelo experimental também para estudos com vírus.

Embora usado com sucesso em inúmeros tratamentos e ensaios com patógenos, ainda falta padronização para o uso efetivo do EG como modelo experimental em especial para teste com fármacos. Alguns autores usaram o EG para teste de toxicidade avaliando peso, alterações histológicas, contagem de células sanguíneas, quantificação de metabólitos em soro e em líquido alantoide e estresse oxidativo [11–16]. No entanto, percebe-se que para cada tipo de experimento com testes com fármacos ou infecção há idades de inoculação e coletas distintas além de vias que podem alterar de um trabalho para outro. Os autores usam normalmente como via a membrana da casca [17], o líquido alantoide [10], a membrana corioalantóide [16], o saco vitelínico [18] e o âmnio [19]. A idade de inoculação também pode ser um fator crucial

nos trabalhos com fármacos visto que o EG tem um desenvolvimento acelerado e um grau de maturidade que altera rapidamente durante as diferentes fases do desenvolvimento. Estudos com vacinação e replicação viral em EG mostram que a via é fundamental para resposta imune e a viremia, mas trabalhos semelhantes não têm sido realizados com fármacos.

O amplo uso de EG como modelo e sua contribuição para a ciência é sem dúvida de grande valor. No entanto, há uma constante falta de padronização do uso desse modelo. Por exemplo, não se entende se parâmetros como contagem e caracterização de células sanguíneas, quantificação de metabólitos em soro e alantoide, alterações histológicas e o estresse oxidativo são variáveis replicáveis de EG a outros modelos como os roedores. Além disso é importante entender se parâmetros como mortalidade, lesões e peso do embrião e seus anexos em EG com diferentes idades podem ser indicadores viáveis em testes com fármacos e infecção. Trabalhos de padronização são necessários para melhor entender a viabilidade dos EG como modelos para testes pré-clínicos.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo é verificar se resultados de viabilidade, peso, alterações macroscópicas, quantificação e caracterização de células sanguíneas, quantificação de metabólicos e minerais em soro sanguíneo e líquido alantóide, alterações histológicas e de estresse oxidativo em EG de diferentes idades são peculiares ao modelo, propondo e discutindo a necessidade de uma padronização tendo como referência um vírus e dois fármacos que causam alterações conhecidas em adultos e outras espécies.

2.2. Objetivos específicos

Avaliar as respostas do EG após o uso de filgrastim (FG) e dexametasona (DX) inoculados na membrana da casca (MC) e membrana corioalantoide (MCA) em diferentes idades;

Verificar parâmetros ainda não estudados em embriões após o desafio com *Gammacoronavirus* de galinha;

Verificar, após o tratamento com os fármacos e o vírus, se há alterações semelhantes nos valores hematológicos e metabólitos séricos e de líquido alantóide;

Observar a interferência da idade embrionária na resposta às variáveis testadas;

Definir a melhor idade para aplicação de inóculos e avaliação das variáveis;

Observar as respostas do EG a determinados fármacos e comparar com respostas produzidas por outros modelos experimentais.

3. REVISÃO DE LITERATURA

3.1. O desenvolvimento do EG

Os ovos das aves possuem tudo o que o embrião necessita para se desenvolver completamente, exceto pelo oxigênio, que penetra no ovo pelos pequenos poros existentes na casca. O vitelo ou gema, é composto por lipídios, proteínas e envolto por uma fina membrana denominada membrana vitelina, que no embrião formará uma bolsa chamada saco vitelínico, próxima da qual fica o pequeno embrião em formação. O albúmen, também identificado como clara do ovo, envolve o vitelo, sendo limitado pela membrana da casca que fica imediatamente abaixo da casca, exceto no polo maior do ovo, onde estão separadas por uma camada de ar, denominada câmara de ar. As chalazas são estruturas que sustentam o vitelo no centro do ovo (Figura 1).

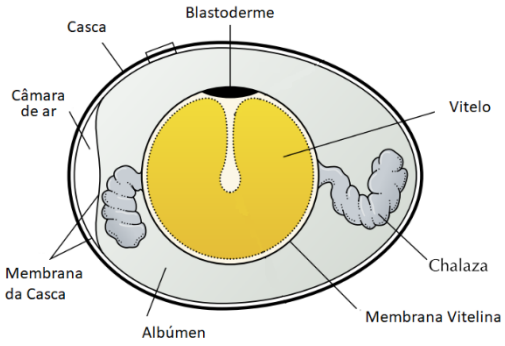

Figura 1. Estruturas do ovo de galinha. Fonte: Bellairs e Osmond (2014)

À medida que o embrião se desenvolve, são produzidos o âmnio, que contém fluido banhando o embrião, o alantóide, no qual restos do metabolismo embrionário são coletados, e o cório, que interage com o ambiente externo seletivamente,

permitindo que determinadas substâncias cheguem ao embrião. O alantóide começa a se desenvolver antes de quatro dias de desenvolvimento embrionário, e sua fusão com o cório para a formação da membrana corioalantoide se inicia entre os dias 6-7. A membrana corioalantóide é a maior das membranas do embrião e envolve a maior cavidade dentro do ovo, a cavidade alantóide que pode conter até 10 mL de líquido alantóide, dependendo do estágio de desenvolvimento embrionário [10] (figura 2).

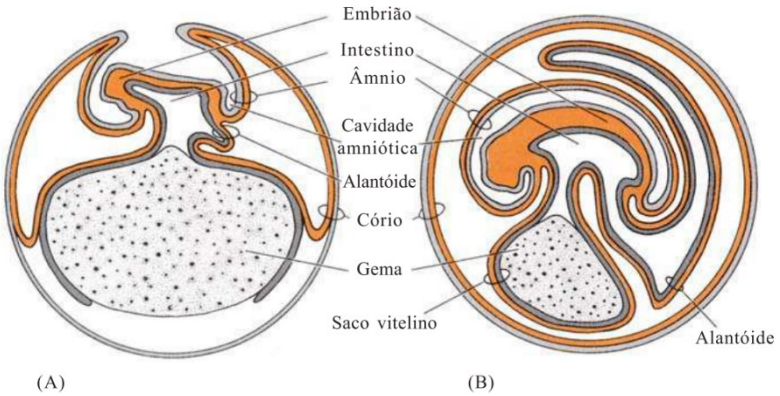

Figura 2. Diagrama do ovo mostrando o desenvolvimento das membranas envolvendo o embrião. (A) Incubação de três dias e (B) Incubação de sete dias. Fonte: Gilbert et al. (1995).

Aristóteles, conhecido como o pai da embriologia, com um simples experimento no qual abria um ovo de galinha a cada dia durante três semanas, observou a formação dos principais órgãos, desde uma fina camada de células até o desenvolvimento completo da ave. As fases deste desenvolvimento, denominado embriogênese, compreendem a clivagem, na qual inúmeras divisões mitóticas dos chamados blastômeros do zigoto resultam na formação da blástula, a gastrulação com a reorganização dos blastômeros em regiões celulares denominadas camadas germinativas, sendo elas o ectoderma, o endoderma e o mesoderma e, por fim, a organogênese, na qual ocorre a interação das células destas diversas camadas para a formação dos órgãos [20].

Para possibilitar a evolução do estudo da embriologia, foi necessário classificar o desenvolvimento embrionário em fases denominadas estádios e o estadiamento embrionário mais utilizado em aves domésticas é o de Hamburger e Hamilton (1951)[21], no qual os autores identificaram 46 estádios distintos em embriões de *Gallus gallus* utilizando características morfológicas externas do embrião como referência. Por meio do estadiamento, é possível identificar e relatar com maior acurácia a fase de desenvolvimento dos embriões observada nos experimentos [1,22].

Após a fertilização do ovo, ainda no oviduto da galinha, inúmeras clivagens dos blastômeros dão origem à blastoderme ou blastodisco, que é uma fina camada de células localizada logo acima do vitelo. Na região central deste disco, os blastômeros centrais, que não tem contato com o vitelo, pois estão situados sobre uma cavidade denominada blastocele, se separam dos blastômeros periféricos, que tem contato com o vitelo, para formar a área pelúcida que é translúcida quando observada de cima, enquanto estes últimos formam um anel periférico mais escuro no qual as células estão sobrepostas, sendo então chamado de área opaca. A camada de células superior a blastocele é o epiblasto que dará origem ao embrião propriamente dito e a camada inferior, o hipoblasto que dará origem às estruturas extraembrionárias[23] (Figura 3).

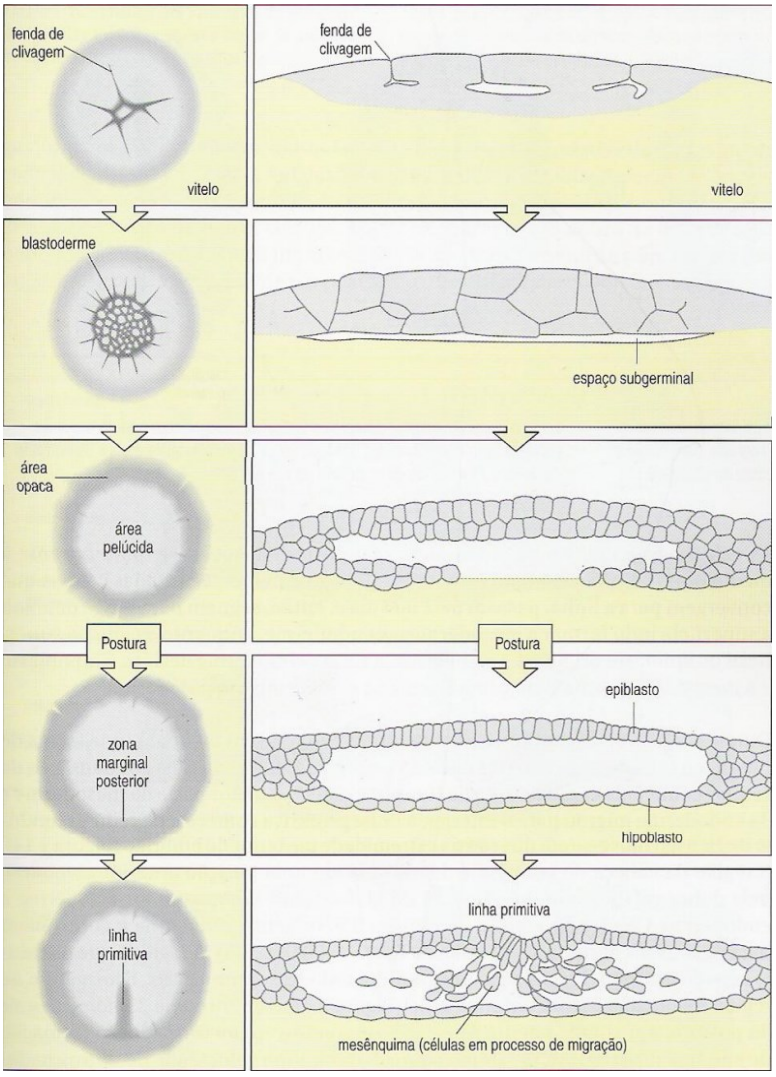

Figura 3. Clivagem e formação da linha primitiva. Fonte: Wolpert, et al. (2000).

Após a postura do ovo, inicia-se a gastrulação, entre os estágios H-H (1-3), que consiste na migração das células do epiblasto para a blastocele dando origem à linha primitiva, que determina o eixo do embrião, inicialmente visualizada como uma faixa mais densa estendendo-se da zona marginal posterior até a região central da área pelúcida, terminando em uma zona mais brilhante, conhecida por nó de Hensen. Durante a formação da linha primitiva a blastoderme perde sua forma circular e se torna mais alongada. As células do epiblasto, proliferando e migrando para o interior do blastocele, vão posteriormente dar origem a células mesodérmicas e endodérmicas[20] (figura 4).

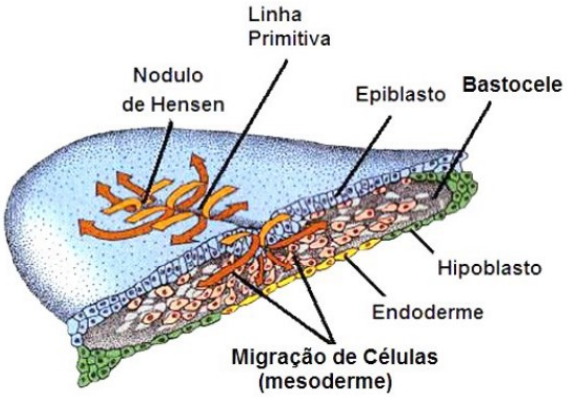

Figura 4. Gastrulação e migração das células do epiblasto para o interior do blastocele através da linha primitiva. Fonte: Gilbert et al. (1995).

Na segunda fase da gastrulação entre os estágios H-H (5-7), a linha primitiva começa a regredir e o nó primitivo move-se em direção à extremidade posterior do embrião. À medida que o nó primitivo regride, ocorre a formação da notocorda e a somitogênese, formação de blocos compactados de células, os somitos, com o aparecimento de um par de somitos a cada hora, aproximadamente. Assim, a adição regular dos somitos durante o crescimento do embrião em idade faz com que o número de somitos seja um critério confiável para analisar seu estágio de desenvolvimento entre os estágios H-H (7-14) [21].

O primeiro órgão a ser formado é o coração, iniciando como um simples tubo por volta do estágio H-H (9-10), aproximadamente 33 horas de desenvolvimento embrionário, e a primeira batida do coração acontece no estágio H-H (10), antes mesmo do estabelecimento da circulação sanguínea que só ocorre a partir do estágio H-H (12-13) [1]. As ilhas de sangue são visíveis no saco vitelínico por volta do estágio H-H (6) e suas células periféricas dão origem ao endotélio e as demais células tornam-

se as primeiras células tronco hematopoiéticas. Os primeiros eritrócitos são encontrados por volta do estágio H-H (7) e a maioria dos vasos sanguíneos embrionários são formados pelo desenvolvimento das células precursoras endoteliais, os angioblastos e este processo é denominado vasculogênese para distinguir da angiogênese. Uma vez que os principais vasos são formados na vasculogênese, inicia-se o processo de angiogênese com a formação de vasos extras, porém como os vasos se diferenciam em artérias ou veias, ainda não está bem elucidado. Células endoteliais da aorta começam a se diferenciar em células hematopoiéticas e colonizam outros órgãos como fígado, baço, timo, medula óssea, Bursa de Fabricius e até mesmo o alantoide. No baço a hematopoise se inicia por volta do décimo dia de desenvolvimento embrionário, apesar das células hematopoiéticas já estarem presentes desde o quarto dia [1,20].

Além do timo, a Bursa de Fabricius é o principal órgão linfóide nas aves. Durante o quarto dia de incubação, a Bursa de Fabricius forma-se como um divertículo do intestino primitivo e seu papel está relacionado ao timo e ao baço. Nos dias 12-13 células migram do fígado, baço, medula óssea e ilhas de sangue do saco vitelínico para colonizar a Bursa de Fabricius e desenvolver seu tecido linfóide e a síntese de imunoglobulinas começa logo depois. O timo começa a se desenvolver por volta do dia 4-5 e as células-tronco linfoides começam a colonizar o órgão a partir do dia 6. Toda a população de células linfoides no timo é derivada de células-tronco, possivelmente do saco vitelino, transportada pelos vasos sanguíneos. Posteriormente, os linfócitos T emigram do timo e colonizam órgãos linfoides periféricos [1,20].

O mesoderma intermediário dá origem inicialmente ao pronefro, depois ao mesonefro localizado mais posteriormente e que permanece ativo durante boa parte da vida embrionária, e finalmente ao metanefro que é totalmente funcional no décimo quinto dia de desenvolvimento. O rim pronefrico é considerado não funcional, começando por volta de estágio 13, ele degenera e desaparece completamente no oitavo dia. O mesonefro funciona a partir do quinto até o 11º dia, quando começa a degenerar e deixa de funcionar no embrião por volta do 15º dia. O divertículo metanéfrico (o futuro ureter) surge da extremidade posterior do ducto metanéfrico pouco antes de sua junção com a cloaca e começa a se formar no final do dia 4. O rim metanéfrico começa a se desenvolver após o ducto metanéfrico entrar em contato com a região posterior da mesoderme nefrogênica, que não foi incorporada ao

mesonefro. O rim metanéfrico é formado em parte a partir da extremidade mais caudal do mesênquima e parcialmente do ducto metanéfrico. O mesênquima dá origem aos corpúsculos renais e os túbulos corticais, bem como a cápsula e estroma do rim e o ducto metanéfrico forma os túbulos coletores e o ureter. No 11º dia de desenvolvimento embrionário, o rim metanéfrico tem todos os túbulos secretores formados e começa a excretar[1].

A região de formação do fígado está intimamente associada com a do coração, e são conhecidas como as regiões cardio-hepáticas. Mas enquanto o coração é uma estrutura inteiramente mesodérmica, o fígado é formado tanto do mesoderma quanto do endoderma. No estágio H-H (12) o componente endodérmico está localizado no assoalho intestinal e é derivado de um divertículo do intestino e no estágio H-H (14), ele invade o mesoderma que formará a matriz do fígado. O fígado primordial é visível no final do segundo dia de desenvolvimento embrionário[1].

3.2. Células sanguíneas

Durante a organogênese, algumas células sofrem longas migrações do seu lugar de origem até sua localização final. Essas células migrantes incluem os precursores das células sanguíneas[20]. A migração das células hematopoiéticas ocorre mesmo depois do processo de desenvolvimento embrionário em diferentes momentos do processo de maturação destas células e não seguem caminhos definidos podendo ocorrer em resposta a eventos fisiopatológicos[22].

As primeiras células do sangue derivam do saco vitelínico, porém essa população de células é transitória. Em estudos realizados por Dieterlen-Lièvre e Martin (1981)[24], foi demonstrado que as verdadeiras células-tronco hematopoiéticas são formadas dentro de nódulos presentes no mesoderma que envolve os principais vasos sanguíneos, como a aorta[20].

O eritrócito maduro normal das aves possui formato oval, citoplasma abundante e núcleo condensado oval e posicionado centralmente na célula. Frequentemente observam-se eritrócitos policromáticos, apresentando basofilia citoplasmática e núcleo menos condensado em relação ao eritrócito normal. Eritrócitos redondos imaturos denominados rubricitos, também podem ser encontrados no sangue periférico[25].

Os leucócitos granulócitos das aves são os heterófilos, os eosinófilos e os basófilos. Os heterofilos são células com citoplasma claro, sem vacúolos e repleto de

grânulos citoplasmáticos eosinofílicos em formato fusiforme e refringente no centro. O núcleo dos heterófilos maduros apresenta poucos lóbulos quando comparado aos neutrófilos dos mamíferos. Os eosinófilos, apresentam grânulos citoplasmáticos arredondados e núcleo lobulado. Os basófilos possuem núcleo oval e não lobulado e grânulos citoplasmáticos basofílico[26].

Frequentemente são observadas células apresentando grânulos de tamanhos maiores que preenchem todo o citoplasma, que por vezes falham no processo de coloração, apresentando-se incolores ou que podem corar em tom azul. A identificação destas células pode variar entre eosinófilos, basófilos e até mesmo células de Mott, uma variação dos plasmócitos. Muitas vezes, esses esfregaços possuem basófilos em coloração normal, sem evidências que indiquem a presença de células de Mott e seus eosinófilos não estão bem corados. Desta forma, estas células acabam sendo identificadas como eosinófilos[26].

Heterófilos imaturos são raramente observados em sangue periférico de aves. Apresentam citoplasma mais basofílico que heterófilos maduros, núcleo não lobulado e grânulos imaturos. Sinais de toxicidade em heterófilos maduros variam de aumento da basofilia citoplasmática, presença de vacúolos, degranulação e degeneração do núcleo. A degranulação é observada pela presença de grânulos eosinofílicos grandes, claros, redondos e pequenos grânulos basofílicos. O número de heterófilos tóxicos presentes pode indicar a gravidade e sugerir a duração de uma resposta inflamatória. Uma pequena quantidade (25% ou menos) pode estar presente em estágios iniciais e aumenta à medida que o distúrbio se torna mais grave[25].

Os leucócitos mononucleares encontrados em sangue periférico de aves são os monócitos e os linfócitos. Os linfócitos geralmente apresentam núcleo redondo e proporcionalmente maior em relação ao citoplasma, que frequentemente aparece como uma pequena faixa ao redor do núcleo. Variam de tamanho, podendo os linfócitos maiores serem confundidos com os monócitos e os linfócitos menores apresentarem citoplasma com pequenas projeções. Os monócitos são as maiores células presentes no sangue periférico das aves e, frequentemente, possuem formato amebóide com abundante citoplasma apresentando vacúolos[27].

Linfócitos reativos são caracterizados pelo aumento do tamanho da célula, aumento da basofilia do citoplasma e presença de cromatina mais frouxa do núcleo. Plasmócitos podem aparecer no sangue periférico e são linfócitos grandes com citoplasma mais basofílico, núcleo maduro, porém excêntrico e nucléolo evidente. Sua

presença é considerada anormalidade. Os monócitos normais apresentam alguns pequenos vacúolos, porém a presença de monócitos contendo grande quantidade de grandes vacúolos é considerado anormal[27].

Nas aves, as células que participam do processo de coagulação são os trombócitos, que derivam de uma linhagem diferente de células presentes no tecido hematopoiético. São células pequenas e redondas, cujo núcleo possui cromatina extremamente condensada e o citoplasma pouco corado em trombócitos maduros, podendo apresentar alguns pequenos grânulos e vacúolos. São diferenciados dos pequenos linfócitos pois esses possuem razão núcleo citoplasma maior e citoplasma homogêneo e de coloração azulada. Assim como as plaquetas em mamíferos, tendem a formar agregados trombocitários. Os trombócitos reativos apresentam citoplasma irregular e formato mais fusiforme em comparação aos trombócitos não reativos. Formas imaturas de trombócitos podem ser visualizadas em sangue periférico de aves[25].

3.3. Uso de embriões em estudos da toxicidade de fármacos

Cada vez mais as indústrias farmacêuticas vêm sendo criticadas pelo uso de modelos animais para estudos de toxicidade e atividade de drogas no organismo. Na prática, a substituição do uso de animais por um sistema de cultura de células *in vitro* não é ideal por causa da necessidade de avaliação da resposta de um organismo como um todo ao medicamento testado. Os embriões de galinha fornecem uma maneira tecnicamente simples de estudar sistemas biológicos complexos com tecidos vasculares bem desenvolvidos, além de permitir alta reprodutibilidade e ser barato e fácil de manusear, sendo reconhecido como um modelo intermediário que pode preencher esta lacuna. Estudos com medicamentos usando embriões de galinha como modelo podem produzir resultados similares gerados em camundongos, podendo ser usados como preliminares aos estudos em mamíferos como forma de economizar tempo, material e animais[6,7], uma vez que experimentos para determinação de dose, nos quais são utilizados um número muito grande de animais para identificação de dose letal e seleção de dose ideal para estudo, podem ser realizados utilizando o embrião como modelo experimental, reduzindo-se assim a quantidade de droga e o número de animais necessários posteriormente em estudos toxicológicos em camundongos[28,29].

Muitos estudos toxicológicos demonstraram que organismos em desenvolvimento são mais vulneráveis a substâncias tóxicas do que os adultos e seus efeitos são por falhas graves no desenvolvimento do embrião, incluindo efeitos teratogênicos[28,29], cardiotoxicidade[15] reduzindo consideravelmente o desempenho do ovo incubado e aumento da taxa de mortalidade[30]. Além disso, o embrião de galinha fornece um modelo adequado para avaliação in vivo da toxicidade, biocompatibilidade, biodistribuição e farmacocinética do medicamento devido à simplicidade, baixo custo, boa reprodutibilidade dos resultados, redução dos aspectos éticos e legais, e a ausência de influência materna na farmacocinética da droga[31].

3.4. Alterações de parâmetros sanguíneos fisiológicos em animais tratados com dexametasona

A dexametasona é um derivado sintético do cortisol e um dos glicocorticoides mais potentes e amplamente usados. Tem ação prolongada e é 30 vezes mais forte que o cortisol. É muito utilizado para tratamento de dor articular em galinhas, enquanto, em altas doses é usado para tratar o choque e reduzir os efeitos da endotoxemia. Pode ser benéfico na fase aguda da inflamação para restringir a produção de citocinas e proteínas de fase aguda[32].

A administração de dexametasona pode causar efeitos imunossupressores semelhantes àqueles gerados por situações causadoras de estresse nos animais. A concentração de corticosteroides no sangue é um indicador conhecido de estresse em galinhas, e o glicocorticoide dexametasona é bem estudado como um indutor de imunossupressão mediada por células. Pode gerar diminuição de órgãos linfóides como Bursa de Fabricius e baço e causar aumento da relação heterófilo/linfócito, com diminuição de linfócitos e aumento de heterófilos[33]. De fato, segundo Jain (1993)[34], a linfopenia induzida por dexametasona em mamíferos é atribuída à atrofia do tecido linfoide, linfólise no sangue, e aumento do deslocamento de linfócitos do sangue para outros compartimentos do corpo, portanto, os linfócitos na circulação sanguínea diminuem. Os glicocorticóides que são liberados quando as aves passam por situações de estresse, causa heterofilia principalmente induzindo o aumento da liberação de heterófilos pela medula óssea para a circulação. Dessa forma ocorre o aumento da relação heterófilo/linfócito[34,35].

3.5. Alterações de parâmetros sanguíneos fisiológicos em animais tratados com filgrastim

Filgrastim é uma citocina da classe dos fatores estimulantes de colônias granulocíticas (G-CSF) e tem atividade biológica idêntica à do G-CSF humano endógeno. Estimula principalmente a ativação, proliferação e diferenciação de células progenitoras de neutrófilos e tem sido utilizada no tratamento de pacientes com várias condições neutropênicas, iatrogênicas e relacionadas a doenças[36].

Há um gene para G-CSF de frango que é expresso na medula óssea e outros tecidos, e pode estimular a proliferação de células da medula óssea de frango[37]. As galinhas não têm neutrófilos, mas em vez disso tem um equivalente funcional, o heterofilo. No frango, esse gene induz a formação de macrófagos e colônias de granulócitos a partir de células da medula óssea, tendo potencial para controlar a função dos heterófilos e aumentar o número de monócitos circulantes[38].

Linhagens de células hematopoéticas e endoteliais compartilham progenitores comuns. Desta forma, diversos trabalhos demonstraram que as citocinas anteriormente consideradas específicas para o sistema hematopoiético afetam várias funções nas células endoteliais, incluindo a angiogênese[39–41]. De fato, a diferenciação do endotélio vascular durante o desenvolvimento embrionário está intimamente ligada ao aparecimento de células hematopoiéticas primitivas, sugerindo que ambas as linhagens celulares compartilham um mesmo progenitor, o hemangioblasto[1].

3.6. Uso de embriões em pesquisas com vírus

Ovos embrionados são utilizados em laboratórios como sistemas hospedeiros para diversos vírus, para propagação desses microorganismos com o propósito de pesquisa ou também para a produção comercial de vacinas[10]. O embrião em desenvolvimento e suas membranas possuem uma diversidade de tipos celulares que são necessárias para a replicação bem-sucedida de diversos vírus[22].

Os estudos com cultivo de diversos vírus em embriões de galinha se iniciaram após os pesquisadores Goodpasture e Woodruff (1932)[42] obterem sucesso ao infectarem a membrana corioalantóide de embriões com herpesvírus, poxvírus e vírus vaccínia, demonstrando que esse tecido vivo promovia um ambiente no qual poderiam ser cultivados vírus de forma estéril. Também foi demonstrado que as lesões observadas na membrana corioalantóide dos embriões infectados eram muito semelhantes àquelas causadas naturalmente pelas doenças. Desta forma, abriu-se

precedentes para que outros testes de diversos vírus fossem realizados, tornando este um modelo fundamental para o estudo da virologia[43]. Atualmente os embriões de galinhas são úteis para testes de isolamento e titulação viral além de tecnologia de vacinas[10].

3.7. Alterações de parâmetros sanguíneos fisiológicos de animais infectados com o *Gammacoronavírus* da galinha

O vírus da bronquite infecciosa (IBV) é um coronavírus do gênero *Gammacoronavírus* (γ-Co V) da família Coronaviridae que afeta galinhas domésticas e se replica nos tecidos do sistema respiratório, bem como em outros tecidos epiteliais incluindo os rins, gônadas e bursa. Filhotes podem vir a óbito diretamente pelo vírus, porém a grande maioria dos óbitos é devido a infecção secundária causada por bactérias. Em animais jovens, IBV causa sintomas como secreções oculares e nasal e letargia, enquanto aves mais maduras não apresentam sintomas graves, embora possa ocorrer graves perdas econômicas em decorrência da baixa produção nas granjas de aves poedeiras ou de corte[44]. Em ovos embrionados a infecção por IBV causa formação de embriões pequenos, curvados, hemorragia e morte.

Além do sistema respiratório, no qual o vírus se replica, a infecção causa alteração patológica nos rins, resultando em nefrite e a avaliação da concentração de ácido úrico no plasma ou soro é amplamente utilizada em aves para a detecção de doenças renais. O fígado é o principal órgão de desintoxicação e sua função pode ser prejudicada pelas propriedades tóxicas de agente infecciosos, o aumento de enzimas celulares hepáticas como alanina aminotransferase (ALT) e aspartato aminotransferase (AST) no soro e/ou plasma é uma perceptível indicação de danos aos hepatócitos[45].

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CAPÍTULO 2

Physiological changes in chicken embryos inoculated with drugs and viruses arouse the need for more standardization for this animal model

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ABSTRACT

Several works have been developed using the *Gallus gallus* embryo as an experimental model to study the toxicity of drugs and infections. Studies that seek to standardize the evaluated parameters are needed to better understand and identify the viability of chicken embryos (CE) as an experimental model. Therefore, we sought to verify whether macroscopic, histopathological, blood count, metabolic changes and oxidative stress in CE of different ages are peculiar to the model. To achieve this goal *in ovo* assay were performed injecting a virus (Gammacoronavirus) and two drugs (filgrastim and dexamethasone) that cause known changes in adults chickens and other animals Embryo response to virus and drug challenges may not occur as expected for adult chickens and even other species. Although macroscopic and microscopic damage may be visible in viral infections, the white blood cell count and inflammation biomarkers did not change. Filgrastim (FG) testing did not result in the expected effects for CE. On the other hand, with Dexamethasone (DX) changes in blood parameters and biomarkers were inherent to the model and depend on the stage of CE development.

Keywords: chorioallantoic membrane, gammacoronavirus, toxicity

1. INTRODUCTION

The embryonated egg is self-sufficient, and its natural development at 37º C and 60% humidity guarantees the maintenance of these animals without more complex means of support. Furthermore, within the egg, the embryo is a highly controlled, accessible, and relatively transparent model in which normal physiology, disease pathology, and the consequences of experimental manipulation can be easily visualized[1], allowing high reproducibility and simplified and economical experiments[2].

Numerous works have been carried out using the *Gallus gallus* embryo as an experimental model for evaluating the toxic effects of drugs and infections. These studies usually evaluated blood, allantoic and amniotic fluid biochemical parameters, erythrocyte morphology, oxidative stress, and histopathological lesions [3–7]. As well as in the evaluation methods and collected materials, there is much variation in the ages and inoculation routes, among which the shell membrane, the allantoid fluid, the chorioallantoic membrane, the yolk sac, and the amnion are widely used [7–11].

Chicken embryo (CE) development is completed in just 21 days, being the fastest among the most used animal models and equal only to the mouse embryo[12]. Thus, on each day of incubation age, many physiological changes can significantly influence the studies and responses that will be evaluated, making the definition of the day of inoculation and collection crucial for realizing an excellent experimental study.

The standardization of parameters, such as the day of inoculation and collection, the inoculation route used, the biological materials collected, and the types of analyses performed are crucial for the experiment's success. Studies that seek the standardization of such parameters are needed to understand better and identify the viability of chicken embryos and *in ovo* studies as models for preclinical tests. Thus, this study aims to verify if the results of viability, weight, pathological and histopathological changes, blood count, dosage of metabolic and oxidative stress in CE of different ages are peculiar to the model proposing and discussing the need for standardization for embryos, using as a reference a virus and two drugs that cause known changes in adults and/or other species.

2. METHODS

This research was performed in the following laboratories from the Uberlândia Federal University: Poultry Egg Incubation, Nanobiotechnology, Biochemistry, and Molecular Biology, Animal Pathology, Veterinary Clinical, and Rodent Vivarium Network. The studies were divided into different phases. The project was evaluated and approved by the Ethics and Research with Animals Committee of the Universidade Federal de Uberlândia (certificated A011/20 and nº 008/21).

2.1. Challenge of CE with *Gammacoronavirus*

The first part of the experiment was carried out in specific pathogen-free (SPF) eggs of *Gallus gallus* challenged with a *Gammacoronavirus,* the IBV (infectious bronchitis virus) that causes embryonic chicken lesions. The CE was incubated in an artificial incubator (Premium Ecológica®) at 37^0C, with 58% humidity, and turning the eggs in a two-hour interval. The eggs were weighed and inoculated at 10 embryonic incubation days (EID). This age was used in the experiment because between 9 and 10 EID, the lesions in CE are distinguished[13]. A total of 4.35 logs viral particles/CE/of IBV (strain Massachusetts, H52) diluted in sterile PBS was inoculated in allantoic liquid from 10 CE. In parallel, four embryos were inoculated as negative controls with sterile PBS (diluent of the vírus).

After 24 hours of incubation, the CE was evaluated to remove the no-specific mortality. At 17 EID, 0.5mL of allantoic liquid was collected. The blood was collected by sectioning the umbilical vessels and divided into two microtubes: one with EDTA K3 for blood cells analysis and the other with clot activator for serum analysis. In the allantoic fluid and serum, there was the quantification of minerals and metabolic. After collecting the blood, the CE was immediately euthanized, weighed, evaluated for macroscopic changes. The liver was collected stored in formalin for histopathological tests and in liquid nitrogen for oxidative stress evaluation.

2.2. Inoculation with Dexamethasone and Filgrastim

2.2.1. Dose of drugs: Pillot test

Before starting a research, we carried out a pilot test to determine the dose of each drug. In this part of the experiment, we used a commercial line of CE, Hy-Line

W36, because SPF birds are mandatory only for research with viruses. The eggs were incubated since zero EID in conditions identical to those mentioned in item 2.1. There is no standardized allometric extrapolation calculation for CE. Then, we usually test the chicken adult dose at 10 EID CE with an average weight of 20g (CE and embryonic annexes). The intention was to find the dose that would not kill the embryo.

In the pilot test, we tested two vias: shell membrane (SM) and chorioallantoic membrane (CAM). We used an approximate dose for a hatched animal as the basis. The 2-4mg/Kg doses were used for DX[14]. According to the drug manufacturer, there is no indication of FG for birds, so we based it on human doses (5mg/Kg). So, the following doses were tested: 4, 0.4, and 0.08µg/CE of DX and 150, 15, 1.5µg/CE of FG being 3 CE per dose via SM and CAM plus the NC for each via totalizing 42 embryos. After 7 days, at 17 EID, the CE were evaluated by macroscopic changes. The dose chosen was based on the group with no expressive mortality or severe lesion in the embryos. To DX, the dose of 0.08µg/CE did not cause gross lesion in all CE, but the 0.4 and 4µg/CE resulted in green allantoic fluid in CE inoculated in SM and CAM. The dose of 1.5, 15 and 150µg/CE of FG did not change the CE. So, the used dose of DX was 0.08µg/CE (~4µg/Kg) and FG was 150µg/CE (~7.5µg/Kg). As the results of embryos inoculated via CAM and SM were identical, in the first moment, the SM route was used because it is easiest via, provides a lower risk of death or lesions, and is an essential route for young or old embryos (which was used in this work).

2.2.2. Test of the drugs in CE

We started the experiment using CE of 12 EID (Hy-Line W36) because they are older CE and their organs and physiologies are more mature than that of a younger CE. The egg incubation conditions were identical to those mentioned in item 2.1. A total of 18 CE was used being 6 CE of the following groups inoculated via SM: (i) treated with 0.08µg/CE of DX, (ii) treated with 150µg/CE of FG, and (iii) NC, only inoculated with water (diluent of drugs). The CE was treated in intervals of 24 hours during 3 days. At 12 EID, the eggs were weighed. After 7 days post-inoculation (pi), at 19 EID, we carried out the collection of blood and liver. We evaluated the weight and macroscopic lesions similar to described in item 2.1 except for allantoic fluid since the amount of this fluid is very low at this age.

We also performed inoculations with FG (150µg/CE) and DX (0.08µg/CE) and a CN group in intervals of 24 hours during 3 days, being 6 or 8 eggs per group, at ages 0, 3, and 7 EID via SM. After 9, 10 and 11 days of incubation, the CE inoculated at 0, 3, and 7 EID respectively were euthanized, weighed and evaluated the mortality and macroscopic lesions. We didn't conduct blood and metabolical or microscopy analysis in the CE inoculated at 0, 3, 7 EID.

Another assay was performed in CE at 10 EID. In this part of the experiment, the via of inoculation for DX was SM. As there were no changes, as expected, in blood granulocyte counts in CE inoculated at 12 EID with FG, and there were no changes in young CE, the via CAM was used to FG in CE of 10 EID. As the FG pathway in humans is subcutaneous and the CAM pathway in CE has direct access to the vessels, we hypothesize that this would be an alternative to test this drug. Thus, it could be possible to assess whether the route could interfere with the results for FG. This part of the experiment was carried out two times, being a total of 18 CE each time, totalizing 36 CE. The groups were divided: (i) CE inoculated with 0.08µg/CE of DX via SM; (ii) NC of DX – CE inoculated with water via SM; (iii) CE inoculated with 150µg/CE via CAM; (iv) NC of FG – CE inoculated with water via CAM. The CE was treated in intervals of 24 hours during 3 days. After 7 days pi, at 17 EID, blood, allantoic fluid and liver, weight and macroscopic lesions were collected, similar to that described in item 2.1. The experiment was repeated at an interval of 15 days, identical to the previous experiment.

2.3. Weigh of the CE and annexes

During the experiments performed, the eggs were numbered and weighed on the first day of inoculation and their weights were recorded. Then, the embryo and yolk were weighed right after collecting blood and allantoic fluid on the day of collection. As the embryo weight is related to the initial egg weight, we performed an adjustment for an initial egg weight of 50 grams, according to Ribeiro et al. (2020)[7]

2.4. Macroscopic evaluations

We checked and counted the CE that died and identified the death date based on embryo development degree. For live animals, we observed their annexes for the presence of circulatory changes, malformation, and color changes. We also checked the embryo by external evaluation and also evaluated the internal organs for circulatory

changes, malformation and color changes and compared the treated groups with the animals in their respective control group.

2.5. Count blood cells, hematocrit, hemoglobin and erythrocyte indices

To determine the hematocrit value (Ht), we filled capillary tubes up to 2/3 with the blood sample. We centrifuged them at 12,000 g for 5 minutes for later reading on a microhematocrit scale.

Hemoglobin (Hb) concentration was measured using cyanmethemoglobin method based in Collier (1944)[15] with modifications using Drabkin's solution and reading by spectrophotometry at the absorbance of 540 nm. The resulting color is of an intensity proportional to the hemoglobin content in the blood. In parallel, we measured the hemoglobin by calculating 1/3 of the hematocrit value.

The total red blood cells (RBC) count was obtained using the Natt & Herrick (1952)[16] solution. So, we performed a manual count in a Neubauer chamber for the hemacytometer method using blood dilution of 1:200. Counting was done in the five diagonal squares of the central reticulum of the chamber. The result was multiplied by 10,000 to obtain the value of erythrocytes per microliter of blood[17].

The total White blood cells (WBC) count and thrombocytes were also performed using the hemacytometer method by diluting the blood with the Natt & Herrick (1952)[16] solution. The leukocytes and thrombocytes were counted simultaneously in the four external reticulum of the Neubauer chamber. The result was multiplied by 500 to obtain the value of leukocytes and thrombocytes per microliter of blood[17]. Two different people counted the cells to confirm the precision of the result. The characteristic of the cells (thrombocytes and eosinophils) was confirmed by cytochemistry.

We calculated the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) using the formulas[26]:

$$MCV = \frac{(Hc\ x\ 10)}{RBC} \qquad MCH = \frac{(Hb\ x\ 10)}{RBC} \qquad MCHC = \frac{(Hb\ x\ 100)}{Hc}$$

2.6. Characterization of blood cells

The blood smear slides were prepared for staining with fast panoptic dye for differentia leukocyte count under an optical microscope (Olympus CX31, 100x

immersion oil). Slides were also stained using Periodic Acid Schiff (PAS) cytochemical technique for identifying thrombocytes and Sudan Black B for identifying eosinophils[18]. Finally, the cells were counted two times in different moments under an optical microscope.

Knowing that serotonin binds to gaseous formaldehyde, we carry out the marking of thrombocytes. We based on Swayne et al. (1986)[19] with some modifications. First, blood smear slides were placed in a box containing formalin and kept for 24 hours at 50ºC. Next, we read the slides in a UV light microscope (EVOS FL Cell Imaging System, Life Technologies Corporation, USA). The diameter of thrombocytes was measured using the ImageJ morphometry program. Slides from healthy and adult chickens (24 weeks of age) of the same line were prepared in parallel as a positive control.

2.7. Biochemical of the serum and allantoic liquid

As the collection of blood from the embryo is not simple and some researchers perform analysis of metabolites in allantois, we perform the analysis in blood and allantois to see if the results are similar. Biochemical analysis of serum and allantoic fluid was performed in an automatic biochemical analyzer (ChemWell® 2910, Awareness Technology). The analytes aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), alkaline phosphatase (ALP), creatinine (Creat), uric acid (UA), calcium (Ca), phosphorus (P) and C-reactive Protein (CRP) (Bioclin®, Minas Gerais, Brazil) were measured in both, serum and allantoic fluid.

2.8. Oxidative stress

Right after euthanasia, the livers of the chicken embryos were removed and immediately stored at −80ºC. The samples were homogenized with 10 mM sodium

phosphate buffer (pH 7.4) and centrifuged at 800× g 4ºC for 10 min. The supernatant was used to quantify the biomarkers of oxidative stress.

2.8.1. Reactive Oxygen Species (ROS)

The samples were incubated with dichloro-dihydro fluorescein diacetate (10 µM) and 5 mMTris-HCl buffer (pH 7.4) for 3 min. After, the fluorescence was measured at 530 nm (excitation in 474 nm).

2.8.2. Lipid Peroxidation

The liver homogenates were incubated with 0.67% thiobarbituric acid (0.67% TBA) and 10% trichloroacetic acid (TCA), for 120 min. Then, n-butanol was added to the samples to remove the organic-phase and the fluorescence was measured at 553 nm, after excitation in 515 nm. The lipid peroxidation was determined using the malondialdehyde (MDA) analytical curve[20].

2.8.3. Sulfhydryl Group

Sulfhydryl group was detected using ditionitrobenzoic acid (DTNB) diluted in 0.2 mM potassium phosphate buffer (pH 8.0). The liver homogenates were incubated for 30 min with 1mM phosphate buffer (pH 7.4) and 10 mM DTNB solution. The presence of sulfhydryl groups was spectrophotometrically detected at 412 nm[21].

2.8.4. Total Antioxidant Capacity

The liver homogenates were incubated with 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tri (2pyridyl) -striazine (TPTZ) and 20 mM ferric chloride at 37ºC for 6 min, at 593 nm. A trolox analytical curve determined the antioxidant capacity and sodium acetate buffer was used as blank[22].

2.9. Histopatology

The fragments of liver fixed in 10% buffered formalin were processed for the preparation of histological slides stained with Hematoxylin and Eosin (HE)[23]. Histopathologic morphology was qualitatively assessed and classified for the presence of inflammation, degeneration, necrosis and circulatory change.

Intracellular fat deposits (lipidosis) were evaluated semiquantitatively and classified by assigning a score relative to the level of lipid deposits in the sample,

according to the histological classification of Brunt et al. (1999)[24] modified by Angulo (2002)[25] with adaptations.

According to Fáncsi (1982)[26], the cytoplasm of hepatocytes from CE has drops of lipids, and the amount of lipid inclusions increases mainly around the twenty-seventh day. Thus, the presence of small vacuoles in the cytoplasm of hepatocytes was considered physiological in the assessment of lipidosis. The scores were evaluated according to the NC and divided into: Score 0 corresponded to normal with discrete lipid deposits, 1 with slight lipid deposits; 2 with moderate lipid deposits; 3 with a marked level of lipid deposition.

In the analysis of the inflammatory process, the number of inflammatory foci and the amount of inflammatory cells in the foci were microscopically evaluated in all the livers of the embryos according the NC. The inflammatory process was classified as: 0 corresponded to normal, with no inflammatory process, 1 – mild, when the number of inflammatory foci was less than 3 with mild inflammatory infiltrate; 2 – moderate, when the number of inflammatory foci ranged from 3 to 5 with moderate inflammatory infiltrate and 3 – marked, when the number of inflammatory foci was greater than 5 with marked inflammatory infiltrate.

As for circulatory alterations, hemorrhage and congestion, they were evaluated semiquantitatively in relation to the NC as: 0 corresponded to normal, with no circulatory alterations; 1 – light; 2 – moderate; 3 – accentuated.

2.10. Angiogenesis

As no injury was observed in CE treated with FG at any dose or route and we noticed an increase in blood vessels in some eggs treated with FG, we performed another experiment adding 5 and 25 times the dose of FG used in the previous investigation. The dose increase was to assess whether doses higher than those used in humans were capable of causing visible damage to embryos and test the hypothesis that FG induces angiogenesis. We tested 34 CE (Hy-Line W36) at 3 EID divided into the following groups: (i) treated with 0.08µg/CE of DX, (ii) treated with 150µg/CE of FG, (iii) treated with 750µg/CE of FG, (iv) treated with 3.75mg/CE of FG, (v) NC, only inoculated with water (diluent of drugs) all by SM route. The CE was treated in intervals of 24 hours during 3 days. At 12 EID, we evaluated the viability and macroscopic

lesions. We evaluated the angiogenesis in CAM using the software ImageJ with the plugin Vessels Analysis[27].

2.11. Statistical Analysis

We assessed whether the data followed normality. In parametric data, we used ANOVA followed by Tukey-test and non-parametric we used Kruskal–Wallis. For the evaluation of two groups, we used t-test to parametric dates and Kruskal-Walis or Wilcoxon to not-parametric dates. Person's correlation test was performed. We considered a 95% confidence interval using the program Graph PadPrism 9.2.

3. RESULTS

3.1. The virus can change blood cells count, calcium and lipid peroxidation in the liver

Of the 10 CE inoculated with *Gammacoronavirus*, 3 died, 6 had lesions and 1 survived without lesions. The injuries, as expected, were dwarf or curled embryos with green liver in one CE, 5 tiny embryos (being 2 CE with the hemorrhagic or enlarged liver, and one with milky allantoic fluid). The presence of the virus led to a change in the weight of live embryos, as expected. Embryos inoculated with the virus had an average weight of 13.41g (+/-2.86), while negative control (NC) weighed 18.17g (+/-1.62) with p=0.0173. We could not weigh the yolk sac of embryos inoculated with the virus because they disintegrated during manipulation. We perform the blood cell count, and the results are described in table 1.

Table1. Blood cells cout and metabolic and mineral values of serum and allantoic liquid in SPF CE treated with *Gammacoronavirus* inoculated at 10 EID.

	NC (blood or serum)*	Virus (blood or serum)*	NC (AL)	Virus (AL)
Ht (%)	21.00 (+/-1.63)[a]	15.60 (+/-2.30)[b]	-	-
Hg (g/dL) (1/3 Ht)	7.00 (+/-0.55)[a]	5.20 (+/-0.77)[b]	-	-
Hg (g/dL) (Cyanmethemoglobin)	8.05 (+/-0.16)[a]	6.23 (+/-0.99)[b]	-	-
Erythrocytes $x10^6/mm^3$	2.05 (+/-0.15)[a]	1.59 (+/-0.23)[b]	-	-
MCH (pg)	35.15 (+/-3.47)[a]	40.08 (+/-9.95)[a]		
MCV (fL)	38.15 (+/-3.47)a	40.08 (+/-9.95)a		
MCHC (g/dL)	96.96 (+/-17.07)a	100.6 (+/-27.12)a		
Thrombocytes $x10^4/mm^3$	2.21 (+/-0.51)[a]	2.75 (+/-2.05)[a]	-	-
Leukocytes $x10^3/mm^3$	8 (+/-2.67)[a]	20.4(+/-22.5)[a]	-	-
Monocytes/mm^3	28 (+/-55)[a]	254 (+/-465.5)[a]	-	-
Lymphocytes/mm^3	650 (+/-730)[a]	2170 (+/-2943)[a]	-	-
Heterophiles $x10^3/mm^3$	7.29 (+/-2.27)[a]	17.6 (+/-18.8)[a]	-	-
Heterophile/lymphocyte	24.09 (+/-18.53)a	28.32 (+/-20.51)a		
Eosinophils/mm^3	00.00(+/-00.00)	00.00(+/-00.00)	-	-
Unidentified granulocytes/mm^3	23.75(+/-47.5)	259(+/-459.1)	-	-
Basophils/mm^3	00.00(+/-00.00)	00.00(+/-00.00)	-	-
UA (mg/dL)	21.76 (+/-23.13)[a]	22.63 (+/-15.17)[a]	18.53 (+/-21.53)[a]	54.30 (+/-29.89)[a]
Creat (mg/dL)	1.42 (+/-1.21)[ab]	0.50 (+/-0.35)[a]	3.21 (+/-1.41)[b]	1.55 (+/-1.11)[ab]
ALP (U/L)	1857 (+/-1108)[a]	2258 (+/-1540)[a]	80.40 (+/-26.37)[b]	55.74 (+/-49.27)[b]
GGT (U/L)	81,53 (+/-55.34)[a]	252.4 (+/-121.5)[a]	210.00 (+/-172.2)[a]	184.00 (119.40)[a]
AST (U/L)	264.00 (+/-40.84)[a]	451.00 (+/-281.1)[a]	200.00 (+/-123.3)[a]	592.00 (+/-283.10)[a]
ALT (U/L)	57.50 (+/-233.6)[a]	92.00(+/-85.73)[a]	63.00 (+/-38.1)[a]	48.00 (+/-22.80)[a]
CRP (mg/L)	24.55 (+/-10.31)[a]	24.75 (+/-9.91)[a]	59.50 (+/-32.55)[a]	84.00 (+/-45.72)[a]
Ca (mg/dL)	95.23 (+/-66.18)[a]	10.23 (+/-8.08)[b]	16.05 (+/-10.48)[b]	16.60 (+/-13.77)[b]
P (mg/dL)	6.82 (+/-3.70)[a]	5.50 (+/-2.96)[a]	19.19 (+/-8.57)[b]	10.76 (+/-6.32)[ab]

Cells count: blood, metabolics and minerals: Serum. The value in parentheses is the standard deviation, Different letters on the same line show statistical difference. Monocytes, MCV: non parametric test. Unidentified granulocytes: Cells not tagged in Sudan Black B or PAS but not having a standard format.

Figure 1 shows the levels of ROS, FRAP, lipid peroxidation and suphydryl groups in the liver of CE infected with *Gammacoronavirus*. In comparison to the negative controls, the virus decreased lipid peroxidation levels ($p < 0.05$) of the embryos.

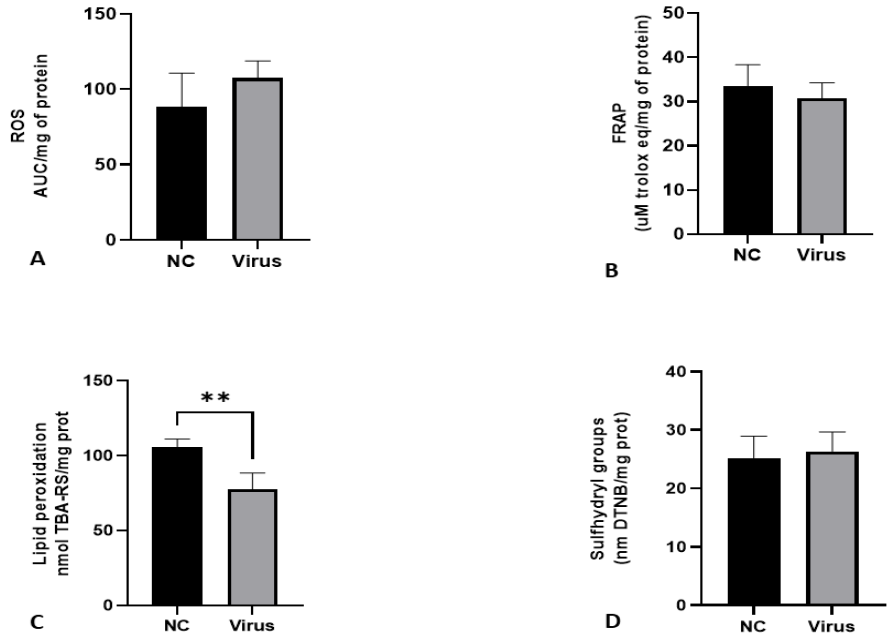


Figure 1. Levels of oxidative stress biomarkers in terms of ROS production (A), FRAP (B), lipid peroxidation (C) and sulphydryl groups (D) in liver of CE after infection with Gammacoronavirus.

In the histopathological analysis of the liver, congestion and hemorrhage were observed in CE infected with the virus. There was also a moderate inflammatory process predominantly composed of heterophils (Table 2, figure 2).

Table 2. Histopathological analysis of liver from CE infected with *Gammacoronavirus.*

	NC	**Virus**
Inflammation	0.00[a] (Mi: 0.00; Ma: 0.00)	2.00 [b] (Mi: 0.00; Ma: 2.00)
Degeneration	0.00[a] (Mi: 0.00; Ma: 0.00)	0.00[a] (Mi: 0.00; Ma: 0.00)
Necrosis	00.00[a] (Mi: 0.00; Ma: 0.00)	0.00 [a] (Mi: 0.00; Ma: 3.00)
Circulatory change	0.00[a] (Mi: 0.00; Ma: 0.00)	1.00[b] (Mi: 0.00; Ma: 3.00)

Mi: Minimum, Ma: Maximum

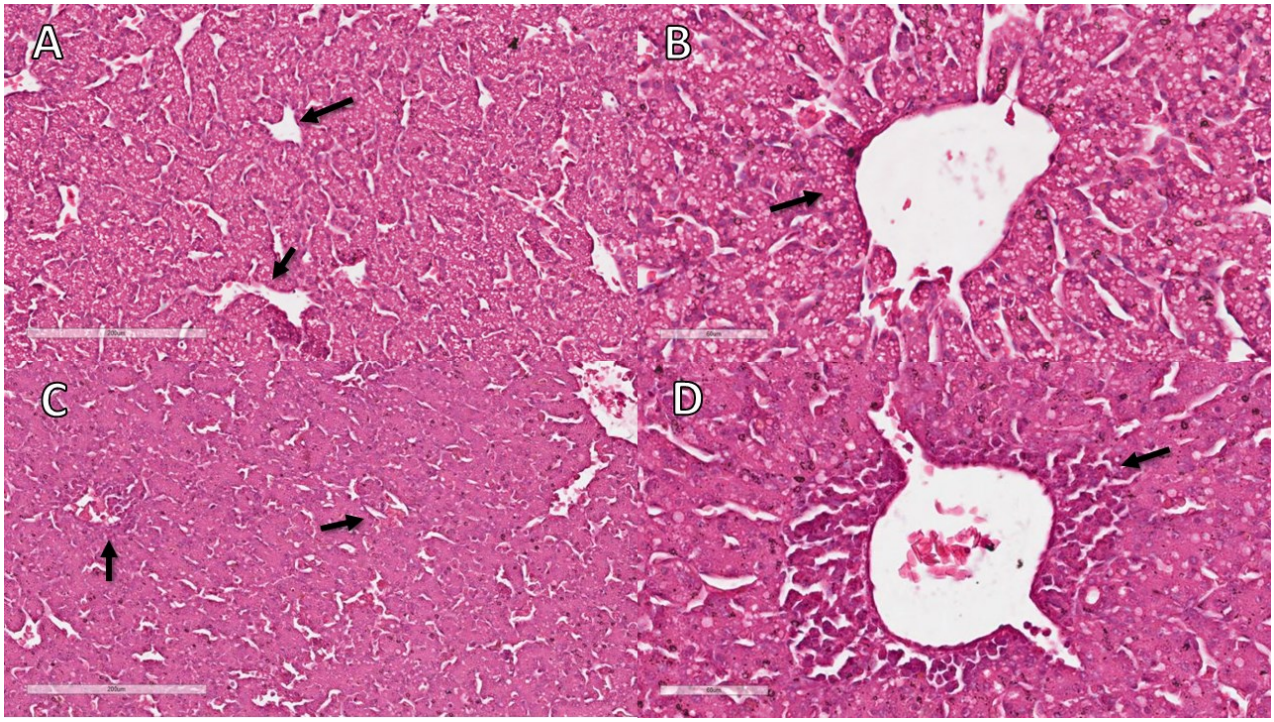


Figure 2. Histopathological analysis of CE liver infected with *Gammacoronavirus* at 10 EID. A. NC sample showing sinusoids with the minimal amount of erythrocytes (arrow). B. NC sample without perivascular inflammatory infiltrates (arrow). C. Liver of virus-infected CE with congestion characterized by erythrocyte-filled sinusoids (arrows). D. Liver of virus-infected CE with inflammatory infiltrate (arrow). The bars in images A and C represent 200µm and the bars in images B and D represent 60µm.

3.2. The age of inoculation and type of drug result in different effects on injury, mortality, or embryo viability

The CE were inoculated with the same dose of drugs at 0, 3, 7, 10 and 12 EID during 3 days. After the inoculation, the CE was evaluated daily by light candling, and the dead CE was necropsied and removed. Between 7-12 days of inoculation, the eggs were opened and necropsied (table 3).

Table 3. Evaluation of CE's viability, lesions, and embryonic mortality treated with filgrastim and dexamethasone in different ages.

	Zero EID			**3 EID**			**7 EID**			**10 EID CAM**		**10 EID SM**		**12 EID**		
	NC	FG	DX	NC	FG	DX	NC	FG	DX	NC	FG	CN	DX	NC	FG	DX
Alive (normal)	6	5	0	6	6	1	6	6	0	5	6	6	3	6	6	3
Injuried	0	0	2	0	0	5	0	0	3	1	1	0	6	0	0	3
Dead	1	1	4	0	0	0	0	0	3	0	0	0	0	0	0	0
Total	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**7**	**6**	**9**	**6**	**6**	**6**

Note: eggs broken or killed by the inoculation process were removed from the analysis. CE inoculated at 0, 3, 7, 10 and 12 EID were evaluated at 9, 11, 10, 17 and 19 EID respectively.

Of the dead embryos inoculated at 0 EID with DX, 2 CE were hemorrhagic and died between 3 to 4 EID; 2 CE died at 9 EID and had a malformation head, and 2 CE survived but had a malformation head. The only dead CE treated with FG died at 9 EID without a specific injury. At 3 EID the CE inoculated with DX had the CAM not wholly formed around the CE, and the yolk sac leaked very quickly. Dead CE inoculated at 7 EID with DX presented smaller size, gelatinous albumen, irregular warping, and green liver, and they died between 14 and 15 EID. Injured live CE showed decreased size and curling, plus petechiae on top of the head. The injured CE in NC and FG inoculated at 10 EID showed a delicate increase in the liver. The injured CE inoculated at 10 EID treated with DX had an enlarged liver and dark or light green. The lesion observed in lived injured CE inoculated at 12 EID with DX was White material over the heart (with uric acid aspect) and beige material within allantois. This material was different in appearance and color from the uric acid commonly found in allantois in normal embryos.

3.3. The weight of CE can be a drug analysis tool depending on age

As shown above, most CE that were inoculated with DX at 0 EID did not survive, so the weights of the embryos from the control group and the FG treated group were evaluated, and there was no difference in weights. CE inoculated with DX at 3 EID showed decreased weight compared to NC and FG groups. The same occurs in CE inoculated at 7 EID. The yolk of embryos inoculated with DX presented a lower weight in relation to the NC and FG groups inoculated at 3 and 7 EID. In CE inoculated at 10 EID and 12 EID, it was observed lower weight of embryos treated with DX compared

to embryos from NC and FG groups, but this difference was not observed in the yolk weights of these animals (Figure 3).

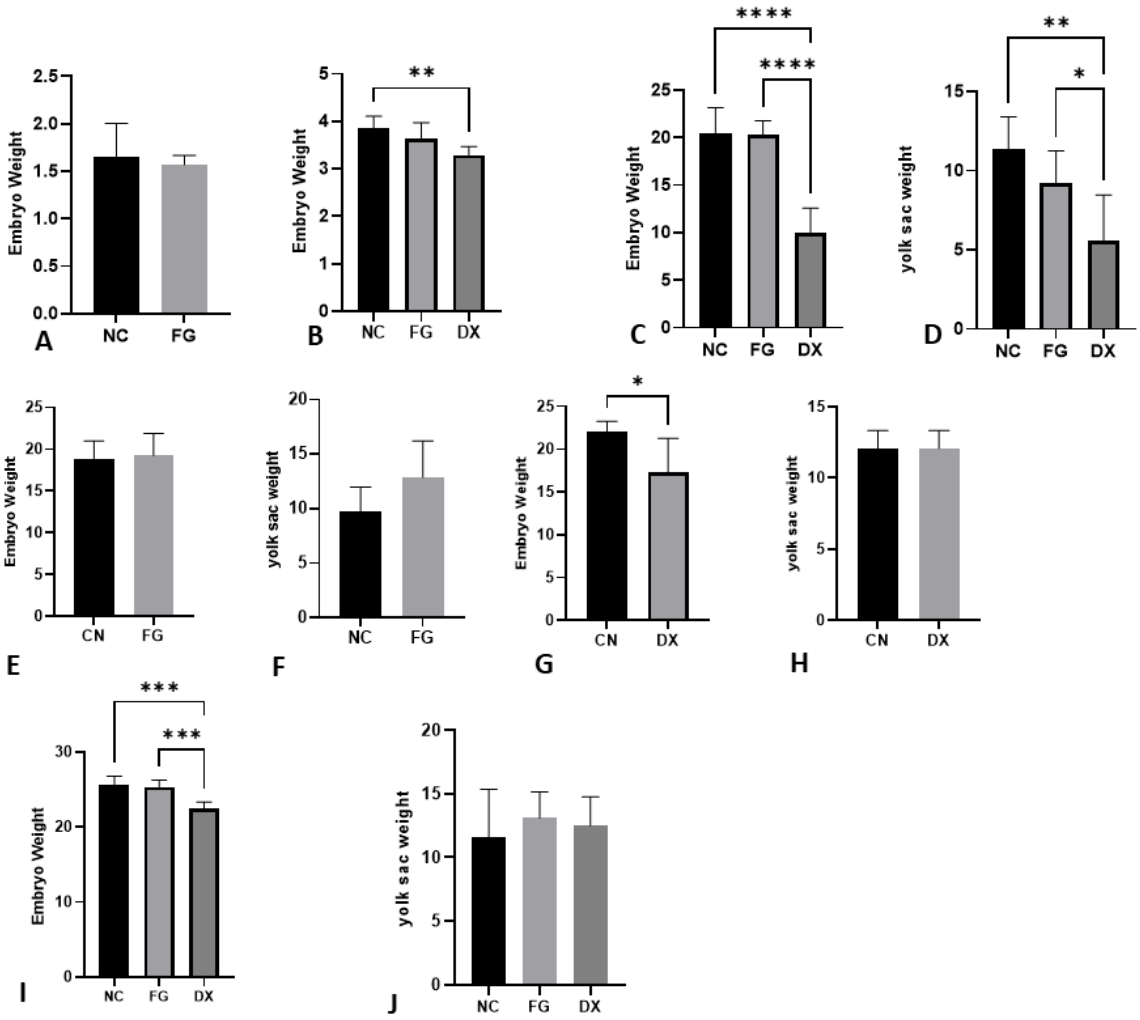


Figure 3. Adjusted embryo and yolk weight in grams (g) at different ages and treatments. Embryo weigh inoculated at 0 EID (A), 3 EID (B), 7 EID (C) inoculated in SM, 10 EID inoculated with FG at CAM (E), 10 EID inoculates with DX in SM (G), 12 EID inoculated at SH (I). Yolk sac weight at 7 EID (D), 10 EID inoculated with FG at CAM (F), 10 EID inoculates with DX (H) in SM, 12 EID (J) inoculated at SH. In CE inoculated at 0 EID the CE of the DX group died. We could not weigh the yolk sac of embryos inoculated with the DX at 0 and 3 EID because they disintegrated during manipulation.

3.4. The blood cell count can change according to the age of inoculation

The blood samples collected from CE treated with FG did not show a statistical difference compared to the NC group for inoculations made at 10 EID and 12 EID, but the same does not occur for CE treated with DX. Compared to the NC group, the samples from embryos inoculated with DX at 10 EID had a smaller number of

erythrocytes, lower values of hematocrit and hemoglobin, and a smaller amount of thrombocytes. No statistically relevant differences were observed in the amounts of total leukocytes. In CE inoculated at 12 EID, embryos treated with DX show a lower number of erythrocytes. The number of thrombocytes was more significant than the NC group, contrary to the CE treated at 10 EID. In the group inoculated at 12 EID also, there was a statistical difference for the amounts of total leukocytes, which was higher for the group treated with DX, Heterophiles which was higher, and Lymphocytes which was lower compared to the NC group (table 4).

3.5. Not all metabolites and minerals change even in liver-damaged embryos

Among the serum and allantoic samples analyzed, only AST from serum samples of CE treated with DX at 10 EID showed a statistical difference compared to the NC group (table 5).

3.6. The amount of metabolites and minerals in serum and allantois are not always identical

The UA, Creat, ALP, GGT, ALT, AST, Ca and P were evaluated of serum and allantoic samples from embryos treated with FG and DX with 10 EID. Only AST and calcium in CE treated with FG and Creat, ALP and P in CE treated with DX showed similar values for the two analyzed samples (table 5). Although the value of the metabolites are not equivalents in all analyses, the results were identical since there were no statistical differences except to AST, which increased in the group treated with DX at 10 EID in serum but not in the allantoic fluid.

Table 4. Blood cell count in CE treated with filtrastim and dexamethasone at different ages.

	10 EID				12 EID		
	NC (CAM)	**FG (CAM)**	**NC (SM)**	**DX (SM)**	**CN (SM)**	**FG (SM)**	**DX (SM)**
Ht (%)	21.64 (+/-3.38)#	18.43 (+/-3.60)#	20.33 (+/-1.86)[a]	17.13 (+/-2.35)[b]	27.80 (+/-3.56)[A]	28.67 (+/-3.50)[A]	23.40 (+/-3.71)[A]
Hg (g/dL) (1/3 ht)	7.20 (+/-1.13)#	6.14 (+/-1.20)#	6.78 (+/-0.62)[a]	5.71 (+/-0.78)[b]	8.93 (+/-1.03)[A]	9.55 (+/-1.17)[A]	9.26 (+/-1.48)[A]
Hg (g/dL) (Cyanmetemoglobin)	8.12 (+/-0.66)#	7.52 (+/-1.29)#	7.83 (+/-0.65)[a]	7.22(+/-1.12)[b]	10.61 (+/-3.04)[A]	10.55 (+/-1.48)[A]	7.90 (+/-1.13)[A]
Erythrocytesx10^6/mm³	1.76 (+/-0.53)[#]	1.73 (+/-0.28)[#]	2.05 (+/-0.27)[a]	1.58 (+/-0.26)[b]	2.59 (+/-0.48)[A]	2.66 (+/-0.38)[A]	1.90 (+/-0.26)[B]
MCH (pg)	50.34 (+/-14.07)#	47.35(+/-4.95)	38.35 (+/-2.04)a	45.58 (+/-6.69)b	41.13 (+/-9.12)A	39.65(+/-2.12)A	41.09(+/-1.39)
MCV (fL)	107.1 (+/-13.82)#	135.0(+/-43.47)#	99.73 (+/-8.53)a	109.10 (+/-13.39)a	102.8(+/-25.82)	108.4(+/-10.98)	128.6(+/-10.29)
MCHC (g/dL)	38.33(+/-3.3)#	41.00(+/-3.48)	38.56 (+/-1.85)a	42.12 (+/-2.65)b	37.75(+/-7.09)A	36.92(+/-4.40)A	33.28(+/-3.18)A
Thrombocytesx10^3/mm³	15.8(+/-0.97)[#]	13.3 (+/-13.1)[#]	16.3 (+/-5.1)[a]	8.75 (+/-5.75)[b]	5.30 (+/-1.35)[A]	5.66 (+/-1.08)[A]	14.5 (+/-7.32)[B]
Leukocytesx10^3/mm³	3.68(+/-2.06)[#]	4.25(+/-2.85)[#]	4.15(+/-0.74)[a]	3.37(+/-2.58)[a]	4.00 (+/-1.05)A	4.96 (+/-1.78)A	16.3 (+/-8.30)B
Monocytes/mm³	0.00 (+/-0.00)[#]	14.17(+/-33.09)[#]	0.00(+/-0.00)[a]	0.00(+/-0.00)[a]	45.83(+/-40.79)AB	61.67(+/-34.86)A	40.00(+/-132.70)B
Lymphocytes* /mm³	159.01(+/-97.72)[#]	220.80(+/-216.80)[#]	318.5(+/-326.3)[a]	357(+/-396.6)[a]	457.60(+/-114.80)A	516.70(+/-139.00)A	271.90(+/-139.80)B
Heterophiles/mm^3	3523(+/-2020)[#]	3973 (+/-2565)[#]	3104(+/-1251)[a]	2453(+/-1695)[a]	3498 (+/-940.50)A	4132(+/-1549)A	16900 (+/-8200)B
Heterophile/lymphocyte	26.26(+/-34.75)#	32.85 (+/-31.77)#	38.51 (+/-38.11)	36.80 (+/-43.29)	8.00 (+/-1.55)A	7.00 (+/-3.30)A	65.77(+/-29.40)B
Eosinophils/mm^3	5.00 (+/-15.00)	25.53(+/-69.48)	0.00(+/-0.00)	1.18(+/-6.03)	0.00 (+/-0.00)	0.00 (+/-0.00)	0.00 (+/-0.00)
Basophils/mm^3	0.00 (+/-0.00)	0.00 (+/-0.00)	0.00 (+/-0.00)	0.00 (+/-0.00)	0.00 (+/-0.00)	0.00 (+/-0.00)	0.00 (+/-0.00)

Statistical comparisons are between FG CAM and NC CAM (test t) or DX SM and NC SM (test t) inoculated at 10 EID (Test t) or between NC, FG, DX inoculated at 12 EID in SM (ANOVA). Different symbols on the same line indicate a statistical difference between FG and NC inoculated at 10 EID via CAM. Different lowercas letters on the same line indicate a statistical difference between DX and NC at inoculated at 10 EID via SM. Different uppercase letters on the same line indicate statistical difference in CE inoculated at 19EID. MCH, eosinophils, monocytes DX 10 EID; Leukocytes, lymphocytes and Monocytes FG 17 EID cam: Non parametric test.

Table 5. Metabolic and mineral values of serum and allantoic liquid in CE treated with filgrastim and dexamethasone at different ages.

	10 EID								12 EID		
	CAM				SM				SM		
	NC (serum)	FG (serum)	NC (AL)	FG (AL)	NC (serum)	DX (serum)	NC (AL)	DX (AL)	CN (serum)	FG (serum)	DX (serum)
UA(mg/dL)	11.00 (+/-7.60)#	16.49 (+/-14.55)#	97.57 (+/-31.16)*	87.88 (+/-51.40)*	13.86 (+/-10.37)ab	12.04 (+/-9.84)a	60.00 (+/-38.63)ab	70.30 (+/-45.93)b	8.28 (+/-1.88)A	12.53 (+/-10.17)A	9.50 (+/-10.82)A
Creat (mg/dL)	1.31 (+/-0.68)#	0.80 (+/-0.63)#	2.49 (+/-0.85)*	3.19 (+/-0.77)*	0.53 (+/-0.35)a	1.12 (+/-1.03)a	3.57 (+/-0.53)b	2.62 (+/-1.29)b	1.37 (+/-1.04)A	0.70 (+/-0.53)A	1.39 (+/-0.53)A
ALP (U/L)	1836 (+/-815,30) #	2380 (+/-298.40) #	47.05 (+/-32.58) *	61.26 (+/-53.26) *	2125 (+/-1365)a	4233 (+/-5830)a	45.94 (+/-39.65)b	77.81 (+/-54.46)b	2194 (+/-322.90)A	2439 (+/-437.70)A	1928 (+/-356.40)A
GGT (U/L)	63.17 (+/-85.13) #	67.00 (+/-81.56) #	14.53 (+/-5.50) #	12.49 (+/-6.98) #	81.20 (+/-94.30) a	49.41 (+/-82.50) a	12.72 (+/-11.31)a	19.51 (+/-9.54)a	47.25 (+/-40.85)A	59.84 (+/-64.05) A	69.08 (+/-47.69) A
AST (U/L)	48.12 (+/-29.53)#	49.18 (+/-15.01)#	15.14 (+/-11.55)*	9.33 (+/-6.02)*	37.55 (+/-20.84)a	99.89 (+/-68.10)b	39.87 (+/-55.81)a	13.27 (+/-4.25)a	123.1 (+/-81.82)A	108.2 (+/-19.21)A	556.1 (+/-27.61)A
ALT (U/L)	54.02 (+/-51.67)# *	158.00 (+/-150.10) #	8.28 (+/-4.07)*	11.71 (+/-8.51)*	142.6 (+/-161.3)a	142.8 (+/-138.7)a	8.00 (+/-1.58)a	13.00 (+/-8.69)a	56.68 (+/-59.33) A	130.5 (+/-226.9) A	73.70 (+/-25.15) Av
Ca (mg/dL)	18.80 (+/-17.03)#	21.80 (+/-21.75) #	12.50 (+/-7.05) #	8.34 (+/-4.62) #	9.15 (+/-10,65)a	30.53 (30.50)a	6.50 (2.80)a	15.91 (13.46)a	17.98 (+/-9.35)A	17.70 (+/-12.28)A	13.63 (+/-10.32)A
P (mg/dL)	7.48 (+/-4.52)#	4.52 (+/-2.58)#	15.37 (+/-9.04)#*	22.13 (+/-9.63)*	4.68 (+/-3.33)a	5.80 (+/-4.21)a	23.65 (+/-6.32)b	26.90 (+/-11.91)b	5.22 (+/-3.89)A	4.76 (+/-2.85) A	9.52 (+/-3.28) A

Statistical comparisons are between FG CAM and NC CAM (test t) serum and AL or DX SM and NC SM (test t) inoculated at 10 EID (Test t) serum and AL or between NC, FG, DX inoculated at 12 EID in SM (ANOVA) in serum because at 19 EID (age of colect) there are not AF. Different symbols on the same line indicate a statistical difference between FG and NC inoculated at 10 EID via CAM. Different lowercas letters on the same line indicate a statistical difference between DX and NC at inoculated at 10 EID via SM. Different uppercase letters on the same line indicate statistical difference in CE inoculated at 12 EID. GGT in DX group inoculated at 10 EID via SM: Non parametric test. GGT and PA in DX group inoculated at 10 EID via SM; Crea group Fg inoculated at 10 EID; UA, ALT, group inoculated at 12 EID: Non parametric test.

3.7. The age of inoculation is an essential factor for changing oxidative stress parameters

Figure 4 shows the levels of suphydryl groups, FRAP, lipid peroxidation and ROS in the liver of CE inoculated with FG and DX at 10 and 12 EID. In comparison to the negative controls, FG increased the levels of sulphydryl groups ($p < 0.05$) of the CE treated t 10 EID. At the same EID, embryos treated with DX increased the levels of ROS production. In CE treated at 12 EID with FG, there was no difference between the NC groups, but the CE treated with DX showed a decrease in the sulfhydryl group and FRAP levels and an increase in the levels of lipid peroxidation and ROS production.

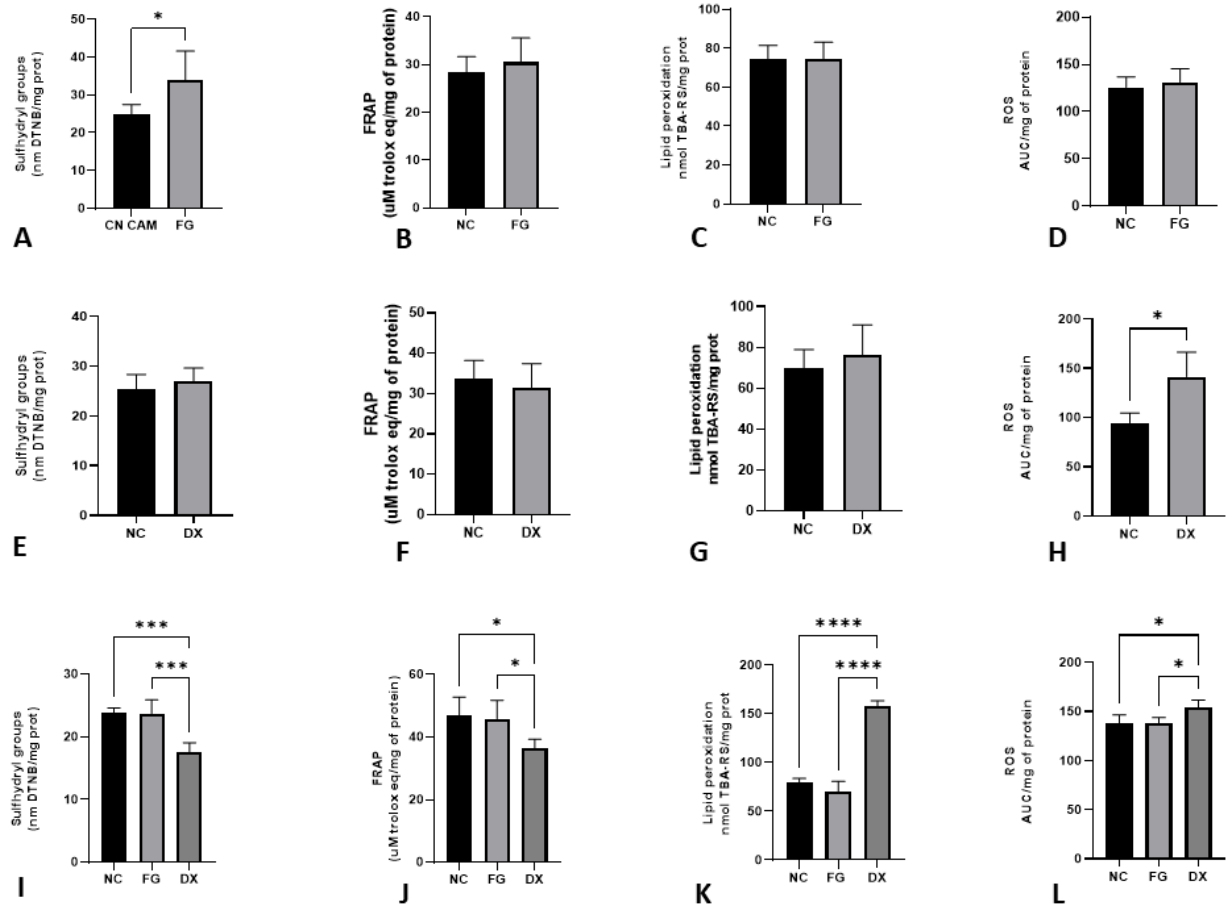


Figure 4. Levels of oxidative stress biomarkers in terms of Sulphydryl groups (A), FRAP (B), lipid peroxidation (C) and ROS production (D) in chicken embryos liver after inoculation with FG via CAM at 10 EID; Sulphydryl groups (E), FRAP (F), lipid peroxidation (G) and ROS production (H) in chicken embryos liver after inoculation with DX via SM at 10 EID; sulphydryl groups (I), FRAP (J), lipid peroxidation (K) and ROS production (L) in chicken embryos liver after inoculation with FG and DX via SM in 12 EID.

3.8. There is a correlation between the results of hemoglobin using a Drabkin solution or performing the calculation of 1/3 of the hematocrit

There was a correlation between the assessment of hemoglobin by calculating 1/3 of the hematocrit and the method using the Drabkin solution. The r-value in the group of CE inoculated at 12 EID was 0.61, representing a moderate correlation [59]. In the groups treated at 10 EID with FG, DX, and vírus, the r-value was 0.86 and 0.85 and 0.86, respectively, showing a strong correlation.

3.9. Characterization of granulocytes and thrombocytes by cytochemistry

Thrombocytes were labeled by the formaldehyde method (figure 5) and the analysis showed that the thrombocytes diameter in CE is lower than in adult animals. Besides, the FG and DX increased the diameter of the thrombocytes.

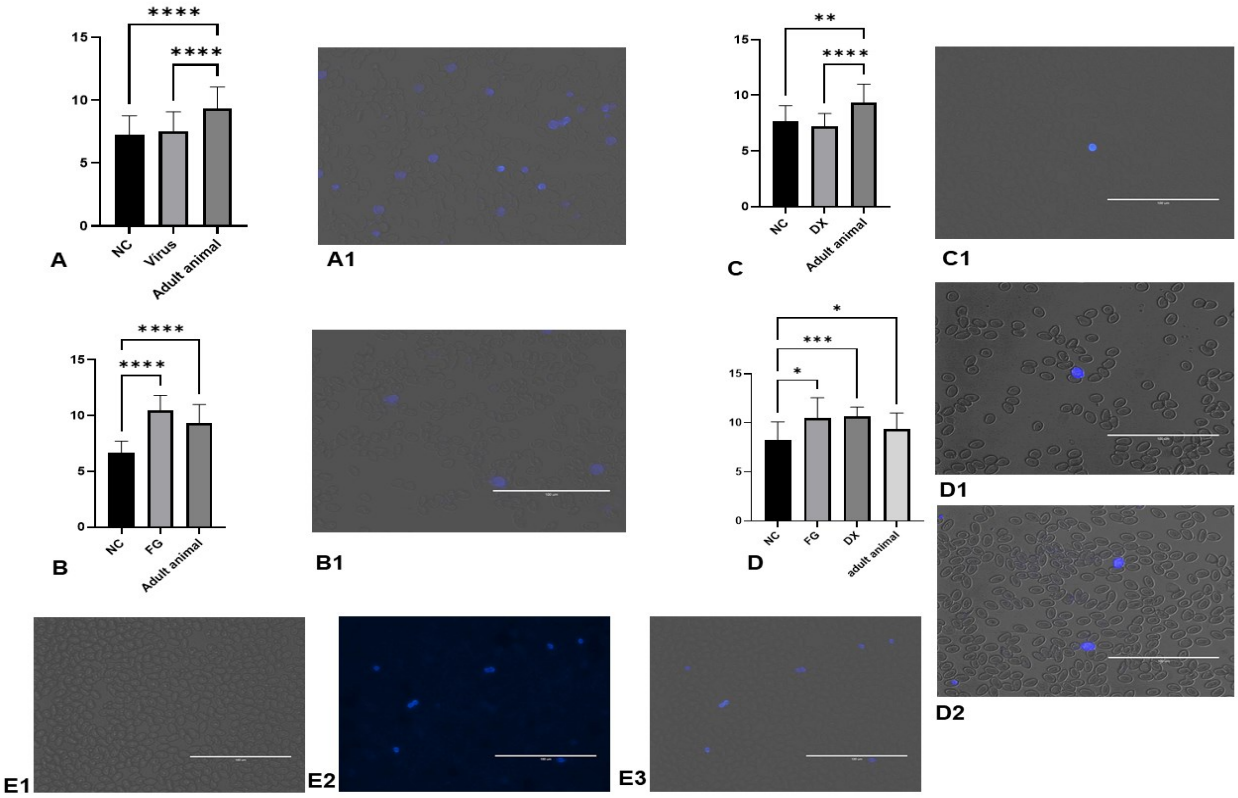


Figure 5. Diameter in µm of formalin-labeled thrombocytes evaluated. A: diameter of thrombocytes in SPF CE inoculated with *Gammacoronavirus*. A1: thrombocytes stained in group treated with virus. B. Diameter of thrombocytes in CE treated with filgrastim at 10 EID. B1: thrombocytes stained in group treated with FG inoculated at 10 EID via CAM. C. Diameter of thrombocytes in CE treated with DX inoculated at 10 EID via SM. B1: thrombocytes stained in the group treated with DX. D. Diameter of thrombocytes in CE treated with FG and DX inoculated at 12 EID via SM. B1: thrombocytes stained in the group treated with FG. E. Thrombocytes stained in the group NC in CE inoculated at 10 EID. E1: trans, E2: UV, E3: over.

Blood smear slides were stained by PAS cytochemical methods for identification of thrombocytes by staining of cytoplasmic glycogen granules and Sudan Black B (SBB) for identification of eosinophils by staining of cytoplasmic granules. These two methods are used for differentiating lymphocytes that are negative for PAS and heterophils that are negative for SBB (figure 6).

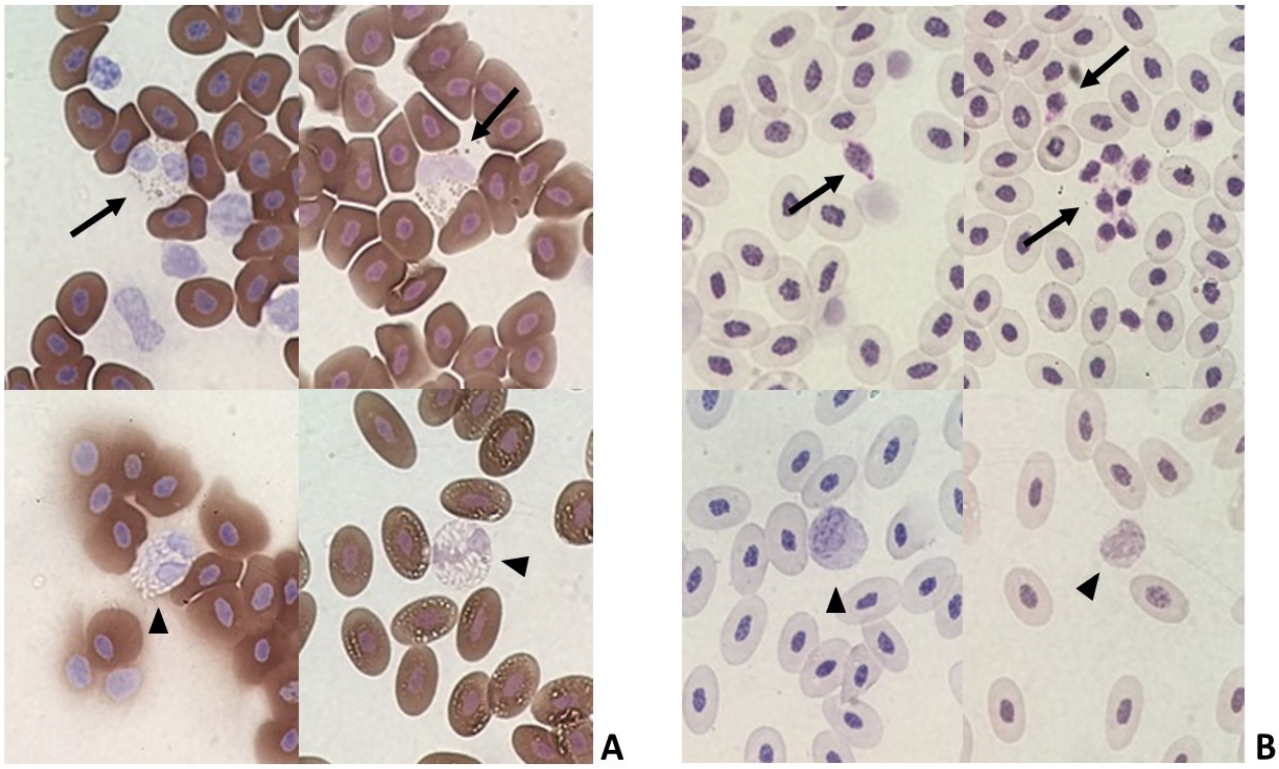


Figure 6. Blood smear slides stained by cytochemical methods. (A) Sudan Black B (SBB) positive eosinophils (arrow) and SBB negative heterophils (arrowhead). (B) Periodic Acid of Shiff (PAS) positive thrombocytes (arrow) and PAS negative lymphocytes (arrowhead).

3.10. Unidentified granulocytes were found in several groups

Despite the various methods of analysis used to identify the cells, some cells with a rounded nucleus and basophilic round cytoplasmic granules were found on some slides in groups NC, FG, DX, and in the group infected with the virus (Figure 7). These cells were not positive by cytochemistry using in our work.

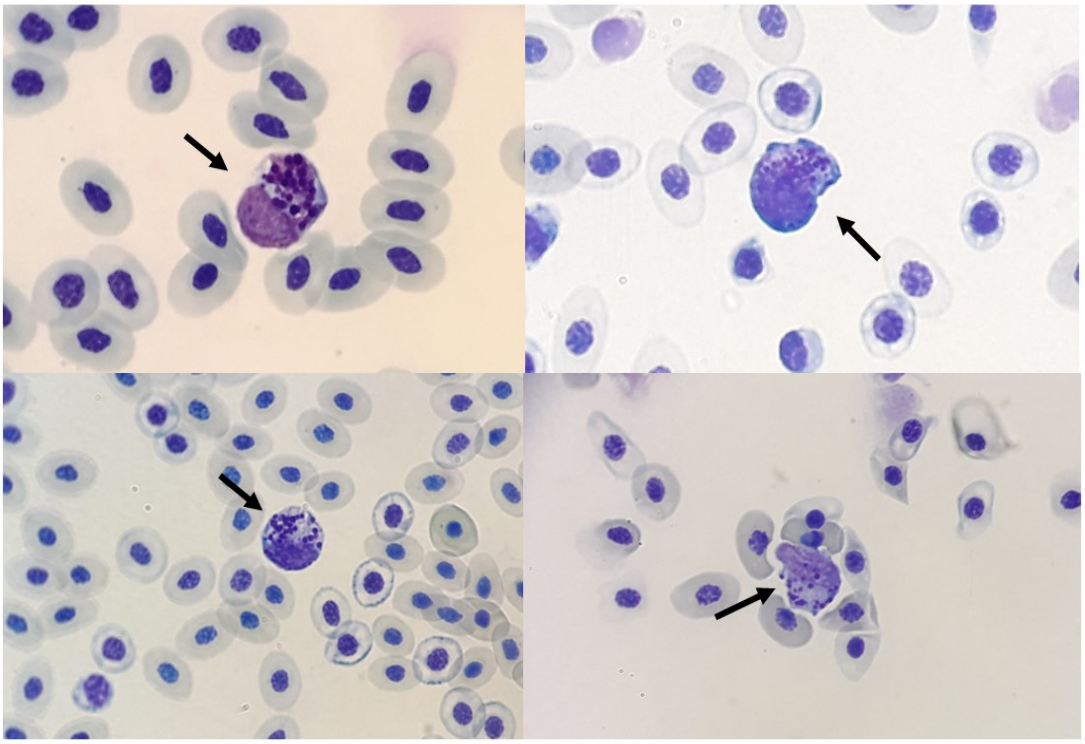


Figure 7. Unidentified granulocytes in blood smear stained with fast Panotico® (arrow 100x).

3.11. Liver histopathological analysis

The degenerative change observed in the CE livers was lipidosis. The hepatocytes were vacuolated, ranging from few and small light vacuoles to large light vacuoles, shifting the nucleus to the periphery, with the hepatocyte resembling an adipocyte. As in virus-infected embryos, the inflammatory process had a periportal distribution and was predominantly composed of heterophils (Figure 8).

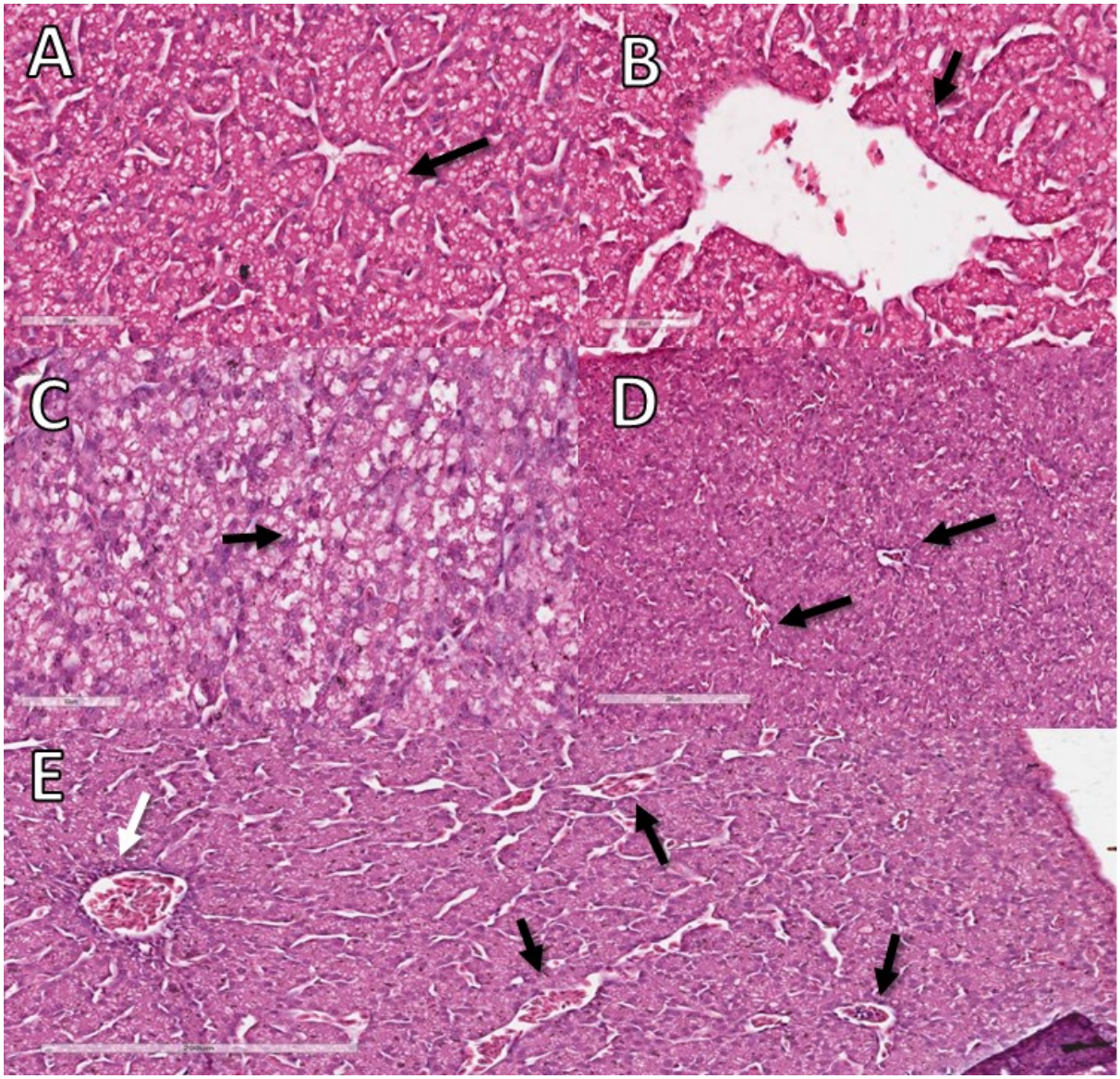


Figure 8. Histopathological analysis of the liver of CE treated with DX and FG in 10 EID. A. CE liver sample from NC with a small amount of lipids in vacuoles in hepatocytes considered normal for comparison with the other groups (arrow). B. Sample from the NC without inflammatory infiltrate (arrow). C. DX-treated CE sample showing large cytoplasmic vacuoles in hepatocytes containing lipid characterizing lipid degeneration (arrow). D. DX-treated CE sample with sinusoids filled with erythrocyte characterizing congestion (arrows). E. FG-treated CE sample with perivascular inflammatory infiltrate (white arrow) and congestion (black arrows). The bars in images A, B and C represent 60µm and the bars in images D and E represent 200µm.

The animals inoculated with FG at 10 EID showed an inflammatory process with infiltration of heterophils. Embryos inoculated with DX at 10 EID showed a mild inflammatory process and circulatory alteration and animals inoculated at 12 EID showed a mild inflammatory process and degeneration (lipidosis) (Table 6).

Table 6. Histopathological analysis of liver from embryos inoculated with FG and DX in 10 and 12 EID.

	10 EID				12 EID		
	NC (CAM)	FG (CAM)	NC (SM)	DX (SM)	NC	FG	DX
Inflammation	0.00 (Mi: 0.00; Ma: 0.00) #	1.00 (Mi: 0.00; Ma: 2.00) *	0.00 (Mi: 0.00; Ma: 0.00)a	0.00 (Mi: 0.00; Ma: 2.00)b	0.00 (Mi: 0.00; Ma: 0.00)A	0.00 (Mi: 0.00; Ma: 1.00) AB	1.00 (Mi: 0.00; Ma: 2.00)B
Degeneration	0.00 (Mi: 0.00; Ma: 0.00) #	0.00 (Mi: 0.00; Ma: 0.00) #	0.00 (Mi: 0.00; Ma: 0.00)a	0.00 (Mi: 0.00; Ma: 3.00)a	0.00 (Mi: 0.00; Ma: 0.00)A	0.00 (Mi: 1.00; Ma: 1.00) AB	1.00 (Mi: 1.00; Ma: 3.00)B
Necrosis	00.00 (Mi: 0.00; Ma: 0.00) #	0.00 (Mi: 0.00; Ma: 0.00) #	0.00 (Mi: 0.00; Ma: 0.00)a	0.00 (Mi: 0.00; Ma: 2.00)a	0.00 (Mi: 0.00; Ma: 0.00)A	0.00 (Mi: 0.00; Ma: 0.00) A	0.00 (Mi: 0.00; Ma: 0.00)A
Circulatory change	0.00 (Mi: 0.00; Ma: 0.00) #	0.00 (Mi: 0.00; Ma: 1.00) #	0.00 (Mi: 0.00; Ma: 0.00)a	1.00 (Mi: 0.00; Ma: 3.00)b	0.00 (Mi: 0.00; Ma: 0.00)A	0.00 (Mi: 0.00; Ma: 1.00) A	0.00 (Mi: 0.00; Ma: 0.00)A

Mi: Minimum, Ma: Maximum

3.12. The FG in high doses don't cause injury o angiogenesis in CE

We tested the hypothesis that FG could cause angiogenesis in CE using several doses. Unlike what we believed, FG could not induce angiogenesis (measured by vascular density and vessel length density), just as DX did not change the number of vessels (supplementary figure 1). As already reported in the previous experiment, DX led to incomplete CAM formation. The high dose of FG also did not cause visible changes in the embryo.

4. DISCUSSION

Chicken Embryos (CE) provides an ideal model for investigating the development of pathogens such as viruses and testing drug effects, evaluating both toxicity and pharmacokinetics. Thus, EC as an animal model can be an important ally for carrying out experiments that require quick and less complex execution and with limited resources

The CE and its annexes constitute a favorable environment for replicating several viruses, being widely used for the isolation or production of vaccines [9]. IBV

causes dwarfism, hemorrhage, and death when inoculated into embryonated eggs. Some strains of the *Gammacoronavirus* can cause nephropathies and other hemagglutination[28]. However, the IBV has a minimal agglutination capacity of erythrocytes in chickens[29]. Our study showed a decrease in hemoglobin concentration, hematocrit value, and the number of erythrocytes in infected embryos compared to NC group, indicating anemia. Hematimetric indices did not differ in relation to the NC group. Thus we can classify anemia as normocytic and normochromic. This means that the cells are in standard size and hemoglobin concentration, so there was a decrease in red blood cells, but there was no response from the bone marrow to release cells in the bloodstream. Normocytic and normochromic anemia can indicate decreased erythrocytes production, which can develop rapidly in birds with diseases involving infectious agents [18].

Although there was no statistical difference in the number of leukocytes in the infected animals compared to embryos in the NC group, some animals had a high leukocyte count (>30,000/mm^3). In contrast, others had a low count (<4,000/mm^3) (supplementary table 1). These results contributed to the increase in the standard deviation in this analysis. The response to the white blood cell count during an infection can vary. In the initial moment of disease, leukocytosis may occur as a response by the body. However, as the leukocytes are consumed, leukopenia may happen when the demand for leukocytes is above the production capacity of these cells by the bone marrow[18]. From 12 EID, the hematopoiesis in CE is more active and happens mainly in bone marrow [30]. So, this work, hematopoiesis in the CE was intense since we used animals between 10-17 EID. However, although the CE used in this work was from the same flock and the mother had high consanguinity, there may be inherent variation in the model itself.

There was no change in the creatinine and uric acid level in serum and allantois in samples from infected CE compared to the NC group, and macroscopic kidney lesions were not observed either. On the other hand, the liver of infected animals was macroscopically altered, with an increase in volume and a greenish color. In addition, we observed moderate inflammatory reaction by histopathologic analyses (Figure 2), although liver enzymes were not increased in serum or allantois compared to the control group[31]. There was also a decrease in lipid peroxidation rather than an increase. The absence of a rise in GGT, ALT, AST, and the reduction in lipid peroxidation indicate that the stress was very intense, reducing liver responsiveness.

Serum calcium levels from virus-infected CE decreased compared to the NC group. Possibly the viruses can hijack the host cell's machinery and utilize the host cell's calcium to create an environment adapted to meet their own demands for replication[32]. This hypothesis is supported in the work of CAO et al. (2011)[33]. These authors observed that the expression level of some calcium-binding proteins was increased after *in ovo* IBV infection. These proteins facilitate the transcellular transport of calcium, suggesting that IBV may disrupt cellular Ca homeostasis for its own benefit.

In addition to virus experiments, drug studies using CE as a model provide a technically simple way to study complex biological systems for *in vivo* drug toxicity and pharmacokinetics assessment. For example, DX is a widely used glucocorticoid. Its administration in CE can cause immunosuppressive effects increasing embryonic catecholamines that alter development and cause death[34].

There was high macroscopic lesion and mortality after the DX inoculation, similar to found by other studies[34–36]. As expected, the mortality was age-dependent. In addition, in our study CE treated with DX reduced the weight in all CE lived. The reduced weight in CE treated with DX can be related to muscle and bone development inhibition[35,36]. Furthermore, high doses of glucocorticoids can generate suppression of growth hormone activity in the pituitary, which is fully established at the beginning of the last week of the embryonic development of CE[36]. Thus, we can suggest that DX promotes delay in embryonic development, negatively influencing embryo weight gain, which may explain the delay in the complete formation of CAM founded by us.

Unlike DX, FG did not cause significant changes and deaths. The only death observed in an embryo inoculated with 0 EID was not accompanied by changes and occurred in an embryo from the NC group, probably caused by a natural process. Likewise, the injury in the CE inoculated via CAM possibly resulted from the inoculation process since the hemorrhage may occur using this route.

CE treated with DX at 10, and 12 EID were anemic. The CE treated with DX showed decreased erythrocyte count and decreased hematocrit and at 10 EID hemoglobin concentration compared to CE in the NC group. There were a decrease in hematocrit, hemoglobin and erythrocyte values also in animals treated with DX in 12 EID, but only erythrocytes showed a statistical difference for the NC group. Despite that, there was a high correlation between hemoglobin and hematocrit (r-value = 0.78), hematocrit and erythrocyte (r-value=0.77) and hemoglobin and erythrocytes (r-value = 0.81). So, the erythrocyte reduction accompanied the reduction in hemoglobin and

hematocrit. Although the CE treated with DX had anemia, the hematimetric indices were not statistically different between the groups treated and NC, defining anemia as normocytic and normochromic. Chickens treated with corticosteroids show increased energy expenditure[37]. Embryos treated with DX at 10 EID had higher yolk consumption, observed by the lower yolk weight of these embryos compared to animals in the NC group. The nutritional deficit associated with liver damage caused by the drug may have impaired the production of erythrocytes, causing normocytic normochromic anemia.

In CE inoculated with DX at 10 EID there was a decrease in the number of thrombocytes compared to embryos from the NC group. Perhaps the production of thrombocytes in CE treated with DX at 10 EID was impaired by the damage caused in the liver. Thrombopoietin (TPO) is the regulator of megakaryocyte development, and thrombocyte production and its expression in chickens occurs mainly in the liver[38]. Hemopoietic activity in the liver starts at 7 EID with a peak at 14 EID[39], but is more active from 12 EID[30]. In animals treated with DX in 12 EID, the opposite was observed. There was an increase in the number of thrombocytes compared to CE from the NC group. Thrombocytosis may reflect a rebound response after recovery from other conditions associated with excessive use of thrombocytes. Considering that CE from 12 EID has a more mature liver, active bone marrow and hemopoietic activity[30], thrombocytosis can be explained in CE treated at 12 EID. Also, it should also be considered that in birds, thrombocytes have phagocytosis function[17]and the influence of glucocorticoids on these cells is not known[40].

In embryos treated with DX at 12 EID, there was an increase in the number of leukocytes, different from that observed in the CE inoculated with 10 EID. This can be explained by the onset of lymphoid activity in the Bursa of Fabricius at 12 EID[41], with a greater capacity to respond to the stimulus caused by the drug. In mammals treated with DX, an initial leukocytosis may occur, mainly due to neutrophilia[29]. In fact, CE inoculated with 12 EID showed an increase of H/L ratio due to the increase in heterophils and a decrease in lymphocytes. This corroborates to several studies in born animals[42–44]. During CE development, granulopoiesis is more predominant; however, at hatching, the granulocytes begin to be replaced by lymphocytes by first 3 days[18].

Taken together, the results show that embryos with a small difference in embryo development stage can completely alter the cell count response. We don't know if this

event happens with other drugs. However, knowing that the embryo is an ascending animal model, further work must be carried out to consider the best age for using the model depending on the expected objective.

As a granulocyte colony-stimulating factor (G-CSF), FG is used in human medicine to increase levels of neutrophils in the bloodstream. Therefore, we expected that the same effect would be observed in the CE in this experiment, increasing heterophils that have characteristics and performance corresponding to human neutrophils. However, no increase in granulocytes was observed in animals treated with FG at 10 EID or 12 EID either by inoculation in CAM or SM. Perhaps this stimulus did not occur because FG is a synthetic compound for human use and may not have the same results in other species.

The analysis of biochemical parameters provides essential data for the assessment of the clinical status of the animal. However, the blood sample collected in research with CE does not always allow this analysis because it's not easy and takes time. So, alternatives samples have been used to assess these parameters, such as amniotic fluid and allantoic fluid[5,7,45]. So, in this work, we compared the biochemical analyzes of serum and allantoic fluid and observed that the values found for the two samples were not always similar. However, the differences observed between groups in serum samples were also observed in allantoic samples, except for AST, which may indicate that this enzyme does not have a good analysis from allantois.

It is known that proteins found in serum have different physical and biochemical properties and change in various physiological and pathological conditions. One of the problems with enzymatic analysis methods is that the reagents were designed to provide the substrate and its optimal concentrations for human plasma, but these variables can change depending on the species. For example, birds have deficient levels of activity of the enzyme ALT in the liver tissue, so in cases of severe liver damage, this enzyme may present normal values. AST activity occurs in multiple tissues, but the main ones are the liver and muscle, being considered sensitive but not very specific in cases of liver problems. GGT activity is increased in all conditions in which hepatocellular damage is present. ALP is associated with the regulation of bird growth, participating in chondrogenic and osteoblastic activities. Thus, physiological variations can be observed, with higher activity levels resulting from bone growth in young birds. Elevations in ALP may be associated with the liver disease even if its activity in this organ is small[26].

In our study, only the AST enzyme of CE treated with DX at 10 EID showed a statistical difference compared to the CN group. However, histopathological findings and results of oxidative stress biomarkers indicate that there was liver damage. At 12 EID there was no increase of AST in CE treated with DX. At this age, just 50% of the CE had a macroscopic injury (table 2) and maybe because of this, the AST level didn't increase. However, the AST maximum value of the group treated of DX at 19 EID was greater than NC (supplementary table 2). Thus, AST may be the best parameter to be analyzed to assess liver function in embryo serum.

High concentrations (up to five times) of uric acid in plasma can lead to precipitation of this acid in the form of crystals, which accumulate in tissues. Situations of hypouricemia are rare and may be related to severe liver damage with a consequent decrease in uric acid production[46]. The excretion of creatinine occurs via the kidneys, but in birds, most creatine is excreted before being converted to creatinine[18]. Thus, increased creatinine concentrations are rare and may occur in severe renal impairment, significantly if filtration is affected[46]. In our study, no changes in uric acid or creatinine were observed in any of the treatments. The macroscopic lesion was not observed as well. This can mean that there was no damage to the CE kidneys. But, as we didn't perform the histopathological analysis of the kidney, we don't rule out the possibility that the high damage caused by the treatment leads to no changes in the biochemical parameters.

Drugs can cause increased production of oxidants and the formation of free radicals, which, by exceeding the body's ability to neutralize and scavenge these radicals, can cause organ damage[47]. Our study showed an increase in oxidative stress biomarkers ROS in the livers of embryos treated with DX at 10 EID and 12 EID and an increase in lipid peroxidation in embryos treated at 12 EID. In adult animals, the corticosteroid can increase oxidative stress[48], but a similar approach was never studied in CE. The increased energy expenditure triggered by high circulating corticosteroid levels might be responsible for the augmented formation of ROS as reflected by increased lipid peroxidation[48]. The body uses enzymatic and non-enzymatic antioxidants to neutralize damage caused by free radicals and minimize excessive oxidative stress. Sulfhydryl groups are an example of essential antioxidants in controlling these processes and protecting against damage. Embryos that were treated at 12 EID showed decreased sulfhydryl groups and FRAP values, indicating that these

embryos had difficulty matching the damage caused by free radicals in the induction of oxidative stress by DX.

Embryos treated with FG at 12 EID did not show changes in oxidative stress biomarkers compared to CE from the NC group. However, in CE inoculated at 10 EID via CAM, FG increased the sulfhydryl group. Filgrastim is a granulocytic colony-stimulating factor (G-CSF). It has biological activity identical to that of endogenous human G-CSF with a free cysteine at position 17 with an ionized sulfhydryl group that is very reactive to free radical oxidation[49]. From this, we can conclude that FG may have shown an antioxidant effect, with possible protection to the embryo against free radicals formed by oxidative stress.

To measure hemoglobin in this experiment, we used Drabkin's solution and compared it with the values obtained using the Drabkin solution method. Our study showed a moderate correlation in embryos treated at 12 EID and a strong correlation in embryos treated with 10 EID[50]. Thus, we can conclude that the calculation based on hematocrit can be used to approximate the hemoglobin value in situations where measurement by spectrophotometry is not possible. However, this replacement is only possible if there is no suspicion of hemolysis, since hemolysis due to problems in the collection or pathological, promotes a decrease in hematocrit without a proportional reduction in hemoglobin.

To perform the differential count of leukocytes in birds, a great difficulty is differentiating between thrombocytes and lymphocytes. Though not identical, the nuclei of thrombocytes and small lymphocytes are too similar to serve as a basis for distinguishing between these two cell types. In the present study, cells in fast panoptic stained CE blood smears with small, round, or oval nuclei with dense chromatin clumps were categorized as thrombocytes if they had cytoplasmic vacuoles and colorless cytoplasm. Cells classified as small lymphocytes had similar nuclei but scant amounts of blue or dark blue cytoplasm without vacuoles. To establish a basis for the categorization of these cells, some cytochemical properties of these cells were compared. The typical thrombocytes were visualized by UV after being exposed to gaseous formaldehyde. Swayne et al. (1986)[19] observed that most of the thrombocytes (99%) had been fluorescent after gaseous formaldehyde treatment and all of the small lymphocytes had been nonfluorescent. This fluorescence resulted from serotonin condensation products. We measured the thrombocytes found in the slides of animals inoculated in this study and compared them with cells found in the blood smear slides

of adult chickens. Thrombocytes from adult chickens had a bigger size compared to the NC group, which averaged 8.5µm. We cannot understand why FG increased the size of thrombocytes.

Using the PAS we characterized the thrombocytes in PAS positive while lymphocytes and erythroblasts were PAS negative (figure 6). The eoshinophils were SBB positive, while the heterophils were SBB negative (figure 6). The use of PAS and SBB is important to better cell classification.

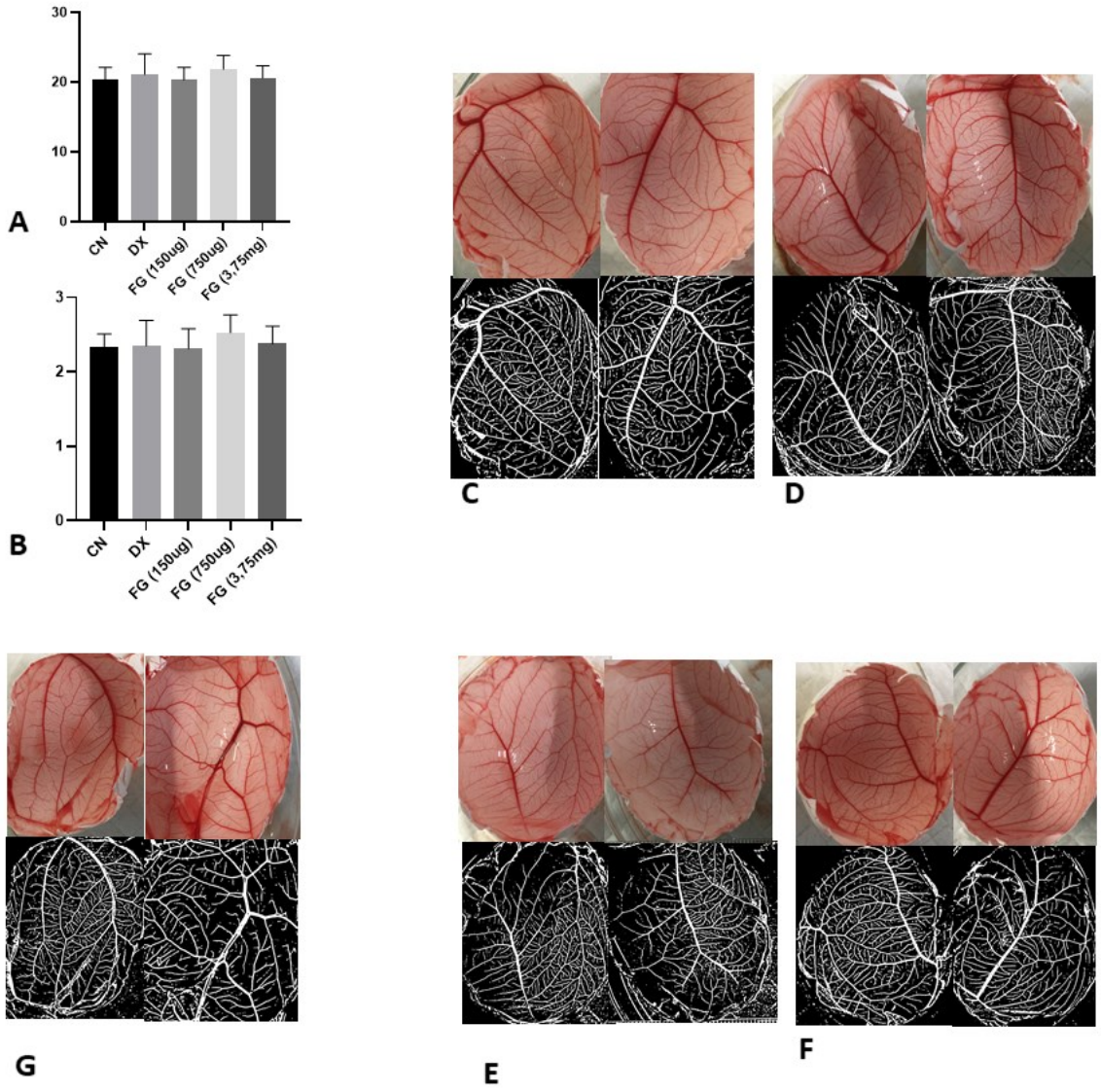
Despite the different forms of differentiation used to identify cells in the blood smear of embryos in this experiment, some cells remained without conclusive identification. The authors identified these cells as a type of granulocyte, but they can be confused with eosinophils, basophils or granulocyte precursors[17,18,51]. These cells were found in samples from embryos treated with FG (20%) and DX (25%) inoculated with 10 EID, animals infected with virus (100%), and in animals from the NC group (23.07%) inoculated with 10 EID.

Another response observed in the use of FG in humans is the stimulation of endothelial cells with consequent angiogenesis[52–54]. In this study, we analyzed the blood vessels of the CAM of animals inoculated with FG to identify a possible increase in vessel density characterizing the occurrence of angiogenesis. However, there was no statistical difference between CE inoculated with FG and the NC group.

Overall, the results of this work provide vital information for the use of CE as an experimental model. The response of CE to challenges with viruses and drugs does not always go as expected. Although macro and microscopic damage were visible in viruses, white blood cell counts and inflammation biomarkers such as C-reactive protein did not change. It is important to mention that some drugs can be innocuous and do not result in expected effects to CE, which was the case with FG. In the case of DX, changes in blood parameters and biomarkers seem to be inherent to the model and are highly dependent on the developmental stage of the CE.

This article reiterates the wonderful value of the CE as an animal model. However, our work sheds light on the importance of standardization and the correct use of the model (considering the laboratory analysis, drug, age, route) so that the infection, toxicity, and pharmacokinetics results are reliable.

SUPPLEMENTAL MATERIAL



Supplementary figure 1. Angiogenesis was measured by vascular density and vessel length density using the software ImageJ with the plugin Vessels Analysis 58. The CE was inoculated at 3 EID and evaluated at 12 EID. A. vascular density, B. vessel length density, C. Photos CAM (NC), D. Photo CAM treated with DX, D. Photo CAM treated with FG (150µg/CE), E. Photo CAM treated with FG (750µg/CE), F. Photo CAM treated with FG (3.75mg/CE)

Supplementary table 1. Maximum and minimum values and median of hematological analysis from CE infected with *Gammacoronavirus* and NC.

	Virus			**NC**		
	Minimum	Maximum	Median	Minimum	Maximum	Median
Hc	13	18	15	19	23	21
Hb	4.33	6.00	5.00	6.33	7.67	7.00
1/3Hc Hb Cyanmetemoglobin	5.02	7.39	6.60	7.70	8.23	8.09
Erithrocytes	1220000	1790000	1680000	1900000	2240000	2020000
MCV	80.81	121.10	92.99	83.33	147.50	87.21
MCH	32.37	42.74	37.70	29.88	58.28	38.40
MCHC	35.00	47.06	39.53	35.86	44.13	39.60
Leukocytes	3500	54000	6000	5500	11000	7750
Heterophils	3325	45360	5640	5280	10340	6788
Limphocytes	70	6480	180	120	1710	385
Monocytes	0	1080	70	0	110	0
Eosinophils	0	0	0	0	0	0
Thrombocytes	9000	51500	19000	18000	29500	20500

Supplementary table 2. Maximum and minimum values and median of metabolites and minerals from the serum and allantoic of CE from CE infected with *Gammacoronavirus* and NC.

		Serum		Allantoic	
		Virus	NC	Virus	NC
UA	Minimum	4.24	1.27	7.15	1.27
	Maximum	36.94	50.07	85.39	50.07
	Median	24.67	17.85	56.93	11.39
Creat	Minimum	0.19	0.26	0.33	1.63
	Maximum	0.96	2.94	2.75	4.92
	Median	0.435	1.235	1.05	3.155
ALP	Minimum	235.7	736.5	18.2	58.2
	Maximum	3845	3388	123.1	82.9
	Median	2476	1651	24.1	68.1
GGT	Minimum	10.2	77.4	40	58
	Maximum	136	356	434	346
	Median	89.6	288	183	166
ALT	Minimum	24	40	20	20
	Maximum	100	220	120	80
	Median	53	54	60	40
AST	Minimum	54	120	120	220
	Maximum	560	780	380	920
	Median	221	452	150	640
Ca	Minimum	2.5	20.5	4.4	10.4
	Maximum	21.5	146.4	39	34.2
	Median	8.45	118.8	14.2	15.8
P	Minimum	2.7	1.4	2.5	11.6
	Maximum	8.4	9.7	18.3	29.4
	Median	5.45	8.1	8.7	17.7
CRP	Minimum	10.8	15	26	40
	Maximum	35.4	38	100	152
	Median	26	23	56	86

Supplementary table 3. Maximum and minimum values and median of hematological analysis from CE treated with FG and DX at 10 EID and their respective NC.

	NC			DX			NC			FG		
	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median
Hc	17.0	22.0	20.5	13.0	21.0	17.5	19.0	28.0	21.0	14.0	25.0	17.0
Hb 1/3Hc	5.67	7.33	6.84	4.33	7.00	5.84	6.33	9.33	7.00	4.67	8.33	5.67
Hb Drabkin	6.48	8.75	7.86	5.16	8.75	7.38	7.08	9.17	8.09	5.58	9.76	7.76
Erithrocytes	1730000	2530000	2012500	1205000	1940000	1537500	1125000	2600000	1772500	1135000	1995000	1760000
MCV	86.96	112.50	98.78	95.74	135.30	108.10	92.39	125.30	104.20	76.92	195.60	138.80
MCH	34.58	40.91		40.98	64.00		31.24	75.29	48.04	36.19	50.04	44.19
MCHC	35.00	41.63	39.04	37.56	47.45	42.71	32.75	42.70	37.63	38.15	49.21	39.63
Leukocytes	1000	5000	4000	500	7500	2500	2000	8500	3000	500	8500	3250
Heterophils	980	4500	3600	495	6300	2300	1780	8160	2880	475	7735	3218
Limphocytes	20	810	210	5	1200	200	60	340	120	25	640	150
Monocytes	0	0	0	0	0	0	0	0	0	0	85	0
Eosinophils	0	45	0	0	210	0	0	0	0	0	0	0
Thrombocytes	11500	22500	14000	1000	15500	8500	5750	34000	14250	2500	39500	7000

Supplementary table 4. Maximum and minimum values and median of metabolites and minerals from the serum and allantoic of CE treated with FG and DX inoculated at 10 EID and their respective NC.

		Serum		Allantoic		Serum		Allantoic	
		NC	DX	NC	DX	NC	FG	NC	FG
UA	Minimum	1.83	0.63	17.52	0.76	2.53	2.64	33.65	17.46
	Maximum	32.13	22.8	115.4	129.9	22.15	38.71	127.8	174.7
	Median	10.82	12.21	53.24	76.43	9.835	7.98	109.4	82.23
Creat	Minimum	0.15	0.12	2.7	1.1	0.41	0.28	1.81	2.16
	Maximum	1.08	3.06	4.26	5.67	2.34	1.98	4.29	4.65
	Median	0.51	0.695	3.735	2.18	1.14	0.51	2.16	3.01
ALP	Minimum	562.6	904.1	9.1	21.5	1114	1830	8.5	9.8
	Maximum	4157	17399	130.9	150.8	3110	2661	98	126.3
	Median	2460	2263	31.9	75	1404	2473	39.1	35.8
GGT	Minimum	5.1	2.4	1.3	4.3	3.1	1.1	7.4	4.7
	Maximum	229	232	27	31	228	204	22.9	21
	Median	14.4	16.4	8.35	17.8	33.3	28	13.95	12.9
ALT	Minimum	14.8	25.8	6	2	5.6	6.2	2	2
	Maximum	437.9	428	10	21	118	364	15	29
	Median	71.4	123	8	14.5	41.95	145.8	8	10
AST	Minimum	11.7	39	5.9	6	15	39.8	3.2	4
	Maximum	63	200.4	31	20.8	74.4	71.5	31.1	17.9
	Median	38.8	79.2	16	14	61.8	42.7	12.6	6
Ca	Minimum	3.1	5.6	3.4	0.8	2.6	3.6	6.3	2.6
	Maximum	30.6	94.2	10.2	41.6	40	60	25.3	14.7
	Median	5.3	20.9	5.8	12.1	16.9	9.8	10.04	6.3
P	Minimum	1.79	0.28	15.6	16.1	2.34	2	4.7	11.1
	Maximum	10.8	13.2	32.5	47.6	15.8	9	29.5	37.2
	Median	3.94	4.03	23.4	20.8	7.8	4.525	15.4	22

Supplementary Table 5. Maximum and minimum values and median of metabolites and minerals from the serum of CE treated with FG and DX inoculated at 12 EID and NC.

		NC	FG	DX
UA	Minimum	5.95	5.72	3.42
	Maximum	10.5	30.18	28.8
	Median	8.7	7.93	5.34
Creat	Minimum	0.32	0.19	0.71
	Maximum	2.76	1.3	1.92
	Median	1.62	0.42	1.47
ALP	Minimum	1754	2071	1528
	Maximum	2566	2980	2391
	Median	2200	2180	1896
GGT	Minimum	9.6	5.6	2.7
	Maximum	104.4	164	108
	Median	37.5	40.2	82.8
ALT	Minimum	4.3	10.6	49.4
	Maximum	150	534	95.4
	Median	58.2	22.5	75
AST	Minimum	57.9	65.9	124.3
	Maximum	259.2	175.8	1319
	Median	112.2	104.4	390.3
Ca	Minimum	9.7	4.1	5.9
	Maximum	32.4	31.5	28.8
	Median	13.8	21.3	9.9
P	Minimum	1.1	1.5	5.9
	Maximum	11.4	9	13.2
	Median	4.4	3.8	9.5

Supplementary Table 6. Maximum and minimum values and median of hematological analysis from CE treated with FG and DX at 12 EID and NC.

	NC			FG			DX		
	Minimum	Maximum	Median	Minimum	Maximum	Median	Minimum	Maximum	Median
Hc	23	31	30	24	34	29	18	26	26
Hb 1/3Hc	7.67	10.33	10.00	8.00	11.33	9.67	6.67	8.67	8.67
Hb Drabkin	8.00	14.08	9.34	8.58	12.13	10.62	6.90	9.76	7.84
Erithrocytes	1900000	3210000	2550000	2020000	3020000	2745000	1600000	2290000	1860000
MCV	80.99	131.60	117.70	89.66	118.80	112.50	113.00	139.80	125.80
MCH	28.23	53.32	42.11	36.07	42.45	39.74	39.37	42.62	41.33
MCHC	31.14	45.43	34.86	31.14	43.58	35.55	30.17	37.54	32.87
Leukocytes	2500	5000	4250	2500	8000	4500	6000	30000	14000
Heterophils	2175	4500	3725	1800	7280	3893	5820	29700	15593
Limphocytes	325.0	650.0	427.5	315	720	571	140	500	220
Monocytes	0.0	100.0	47.5	0	100	75	0	0	0
Eosinophils	0	0	0	0	0	0	0	0	0
Thrombocytes	4000	7500	5000	4000	7000	5750	6000	23500	15000

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