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ANÁLISE DE EXPRESSÃO DE GENES RELACIONADOS À SENESCÊNCIA CELULAR EM EMBRIÕES DE GALINHA COM MICROCEFALIA INDUZIDA POR ZIKV

Porto Alegre
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Orientador: Prof. Dr. Lucas Rosa Fraga
Coorientador: M.Sc. Miriãn Ferrão Maciel Fiuza

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Agradeço a eles,
meu pais,
meus avós,
minha irmã.

Karina Maria Wermann

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RESUMO

O vírus Zika (ZIKV), transmitido principalmente pelo mosquito *Aedes aegypti*, emergiu em 1947 e alcançou notoriedade durante o surto no Brasil em 2015, associado a um aumento de microcefalia em bebês nascidos de mães infectadas durante a gravidez. A microcefalia é a característica primária da Síndrome Congênita do ZIKV (SCZ), cujos mecanismos teratogênicos e impactos no desenvolvimento embrionário ainda são pouco compreendidos. A literatura sugere a participação de genes relacionados ao ciclo celular e desenvolvimento embrionário nesses processos. A senescência celular (SC), um estado em que as células saem do ciclo celular sem entrar em apoptose e adquirem o fenótipo secretor associado à senescência (SASP), apresenta-se como um possível mecanismo relacionado na teratogênese do ZIKV. Um estudo recente destacou associação entre senescência celular e anomalias no sistema nervoso, motivando investigações sobre a indução de senescência celular pelo ZIKV. Utilizando embriões de galinha como modelo, este estudo analisou a expressão gênica de marcadores moleculares de senescência celular em encéfalos de embriões infectados com ZIKV e controles. Aplicou-se 50 μL de meio de cultura ou meio de cultura e 3x10^4 PFU de ZIKV em embriões no estágio HH11-12, incubados por 24 ou 96 horas. Avaliou-se a expressão dos genes *CDKN2A, CDKN1A, CDKN2B, TP53, IL1B, IL6* e *MMP9*, não observando diferenças significativas em *CDKN1A* e *MMP9*. A expressão basal dos demais genes não foi obtida. A complexidade desses mecanismos destaca a necessidade de investigações adicionais para compreender a relação entre a senescência e a teratogênese do ZIKV.

Palavras-chave: ZIKV, microcefalia; Fenótipo Secretor Associado à Senescência (SASP); ciclo celular; anomalia congênita.

ABSTRACT

The Zika virus (ZIKV), primarily transmitted by the Aedes aegypti mosquito, emerged in 1947 and gained notoriety during the 2015 outbreak in Brazil, associated with an increase in microcephaly in infants born to mothers infected during pregnancy. Microcephaly is the primary characteristic of Zika Congenital Syndrome (ZCS), whose teratogenic mechanisms and impacts on embryonic development are still poorly understood. Literature suggests the involvement of genes related to the cell cycle and embryonic development in these processes. Cellular senescence (CS), a state in which cells exit the cell cycle without undergoing apoptosis and acquire the senescence-associated secretory phenotype (SASP), emerges as a possible mechanism related to ZIKV teratogenesis. A recent study highlighted the association between cellular senescence and anomalies in the nervous system, prompting investigations into the induction of cellular senescence by ZIKV. Using chicken embryos as a model, this study analyzed the gene expression of molecular markers of cellular senescence in brains of embryos infected with ZIKV and controls. 50 µL of culture medium or culture medium containing 3x10^4 PFU of ZIKV was applied to embryos at the HH11-12 stage, incubated for 24 or 96 hours. The expression of genes CDKN2A, CDKN1A, CDKN2B, TP53, IL1B, IL6, and MMP9 was evaluated through cDNA from the brain, with no significant differences observed in CDKN1A and MMP9. The basal expression of the remaining genes was not obtained. The complexity of these mechanisms underscores the need for additional investigations to understand the relationship between senescence and ZIKV teratogenesis.

Keywords: ZIKV, Microcephaly, Senescence-Associated Secretory Phenotype (SASP); cell cycle; Congenital Anomalies.

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1. INTRODUÇÃO

1.1 O ZIKV E A SÍNDROME CONGÊNITA DO ZIKA VÍRUS

O vírus Zika (ZIKV) é um arbovírus da família Flaviviridae de RNA de fita simples, transmitido majoritariamente através da picada de artrópodes hematófagos, em específico os mosquitos *Aedes spp.* Esse vírus foi identificado pela primeira vez em macaco Rhesus, na Uganda, no ano de 1947 durante vigilância de rotina contra a febre-amarela, posteriormente foi detectada em humanos em países da África (Lanciotti *et al.*, 2007). Apenas no início do século 21 é que surgiram os primeiros relatos de epidemias, inicialmente em ilhas do Pacífico e posteriormente, no início de 2015, um grande surto no Brasil (Schuler-Faccini *et al.,* 2016). Enquanto em outros países as infecções relatadas apresentavam um impacto relativamente baixo e não letal, o ZIKV tomou notoriedade quando uma epidemia no Brasil levou a um aumento no número de casos de microcefalia em bebês nascidos de mães infectadas (Cugula *et. al*., 2016; Komarasamy *et al*., 2022). Esse fato levou a Organização Mundial da Saúde (OMS) a declarar em 1º de fevereiro de 2016 a infecção por ZIKV uma Emergência de Saúde Pública de Importância Internacional (WHO, 2016).

Os casos de infecção por ZIKV iniciaram na região nordeste do Brasil no início de 2015 e logo observou-se uma dispersão para as outras regiões do país e além das fronteiras brasileiras (Sampaio *et al.,* 2019). Associado a estes eventos, observou-se um aumento expressivo de notificações de microcefalia em bebês recém-nascidos e registros de casos de abortamentos espontâneos e natimortos, gerando uma grande preocupação nos órgãos de saúde pública do país (de Oliveira, *et al.,* 2016). Através de testes de identificação viral no líquido amniótico de gestantes e detecção de anticorpos em 12 crianças com microcefalia pode-se obter uma forte correlação que posteriormente levaram a uma relação causal entre ZIKV e microcefalia (Rasmussen *et. al.,* 2016; Schuler-Faccini *et al.,* 2016). As anomalias observadas em bebês expostos ao ZIKV durante o desenvolvimento embrionário e/ou fetal fazem parte da Síndrome Congênita do Zika Vírus (SCZ), marcada, principalmente, pela ocorrência de microcefalia (Schuler-Faccini *et al.,* 2022).

A microcefalia é uma anomalia congênita caracterizada por um desenvolvimento anormal do encéfalo, no qual este é significativamente menor do que o normal. Ela pode ser causada por infecções congênitas, como é o caso do ZIKV, mas também através de outras

infecções e substâncias que, estando presente na vida embrionária ou fetal, produzem uma alteração na estrutura ou função do embrião em desenvolvimento, como é o caso do álcool, drogas e compostos farmacológicos. Substâncias, infecções e condições maternas que levam às anomalias congênitas são conhecidas como agentes teratogênicos. As consequências da microcefalia podem ser diversas, desde atrasos leves no desenvolvimento como deficiência intelectual, até paralisia cerebral, epilepsia, dificuldade de deglutição e anomalias dos sistemas visual e auditivo (Schuler-Faccini *et al.,* 2022). Além da microcefalia, a SCZ é caracterizada por uma variabilidade de fenótipos, incluindo acentuada protuberância óssea occipital, fontanelas fechadas ao nascer, excesso de pele no escalpo, ossos parietais também deficientes, contraturas significativas de pelo menos uma articulação, alterações da atividade motora (tônus, postura e motilidade), neurocomportamento alterado e excitabilidade além de hérnia umbilical (Del Campo *et al.,* 2017; Schuler-Faccini *et al.,* 2022). É importante notar que estes fenótipos nem sempre ocorrem em conjunto no mesmo bebê e que crianças que foram expostas ao ZIKV durante a gestação podem não desenvolver a SCZ. Esta variabilidade é um dos princípios da teratogênese.

Apesar da significativa redução no número de casos de infecção pelo ZIKV em comparação com o pico durante o surto de 2015, o vírus persiste no país até os dias atuais. De acordo com a Organização Mundial da Saúde (OMS) juntamente com Organização Pan-Americana da Saúde (OPAS), em 2022 houve 11,298 casos confirmados de infecção por ZIKV na América do Sul, sendo destes, 826 no Brasil (PAHO, 2022). As arboviroses são um grande problema mundial pois seus vetores se encontram em praticamente todos os continentes, mesmo que o mosquito tenha uma distribuição geográfica e climática restrita, devido às rápidas mudanças climáticas, os desmatamentos em grande escala, a migração populacional acentuada e facilitada pela globalização, e a precariedade das condições sanitárias em muitos países, há um risco na amplificação e transmissão viral facilitada mundialmente, levando a um risco iminente de novos surtos (Kraemer *et al.,* 2015).

Ainda hoje são escassos os conhecimentos sobre a SCZ, tanto sobre sua evolução natural, como dos seus fatores de risco associados. Evidências sugerem que a transmissão viral da mãe para o feto ocorre por via transplacentária, através da infecção de células Hofbauer (macrógafos placentários) que tem capacidade migratória, e trofoblastos placentários (Komarasamy *et al.,* 2022). Além disso, estudos mostram que o vírus altera a expressão gênica antiviral e de genes associados à resposta imune, desregula o ciclo celular, ativa resposta inflamatória e apoptose em células progenitoras neurais, o que resulta em

malformações neurológicas e mostra que diferentes mecanismos podem ser responsáveis pela neuropatogênese do ZIKV (Faizan *et al.,* 2016; Komarasamy *et al.,* 2022; Schuler-Faccini *et al*., 2022). Sabe-se que a SCZ tem relações com regulação negativa de genes relacionados ao desenvolvimento neurológico e regulação positiva de genes relacionados àresposta inflamatórias. Além disso, estudos tem demonstrado que processos associados ao ciclo celular são afetados durante a infecção por ZIKV, apontando-os como potenciais mecanismos pelos quais o ZIKV leva à ocorrência da SCZ (Schuler-Faccini et al., 2022).

1.2 SENESCÊNCIA CELULAR

A senescência celular é caracterizada pela parada da replicação da célula, e é provocada em resposta a diferentes mecanismos como: disfunção mitocondrial, encurtamento dos telômeros, radiação, agentes quimioterápicos, estresse oxidativo, estresse genotóxico, e outros danos ao DNA (Herranz *et al.,* 2018). As células que entram em senescência permanecem metabolicamente ativas, mas param de responder a estímulos mitogênicos, sofrendo mudanças dramáticas na estrutura da cromatina, na expressão gênica e em sua morfologia (Bem-Porath *et al.,* 2005; Chen *et al.,* 2006). Historicamente esse mecanismo é visto como algo intrínseco à célula e projetado para restringir a proliferação celular ilimitada, causando supressão da proliferação de células tumorais, por exemplo, ou também associado à determinadas condições tais como Doença Hepática Gordurosa não Alcoólica (DHGNA), diabetes, hipertensão pulmonar, osteoartrite, tumorigênese e infecções. Entretanto, seus mecanismos e o papel fisiológico permanecem pouco compreendidos (Wei *et al.,* 2018).

Como descrito anteriormente, a senescência é desencadeada por insultos intrínsecos (dano oxidativo, atrito dos telômeros, hiperproliferação) ou externos (radiação ultravioleta, irradiação, drogas quimioterápicas) que levam a uma resposta ao dano DNA (*DDR*, do inglês *DNA damage response*) (Herranz *et al.*, 2018). Estudos atribuíram a vários genes supressores de tumor um papel crítico nos mecanismos da senescência, envolvendo a maquinaria de verificação do ciclo celular (Chen *et al.,* 2006). A comunicação entre os fatores associados à *DDR* e à maquinaria do ciclo celular é realizada pela fosforilação e ativação de várias proteínas do ciclo celular, que levam à ativação das vias supressoras de tumor p53/p21 (Cip1) e p16 (INK4a)/Rb (Birch *et al.,* 2020; Kuilman, *et al.,* 2010).

A transição de uma fase do ciclo celular para outra é regulada por diferentes proteínas celulares, principalmente quinases dependente de ciclinas (CDK, do inglês *cyclin dependent*

kinase) e a atividade de CDK pode ser neutralizada por proteínas inibidoras do ciclo celular, chamadas de CDK inibidoras (CKI) (Vermeulen *et al.*, 2003). No ciclo celular a parada do ciclo induzido por dano é dependente da p53, e esta tem função relevante na senescência pois estimula a transcrição de p21 (codificado pelo gene *CDKN1A),* um CKI, que inibem os complexos G1 CDK-ciclina e CDK1-ciclina B, interrompendo o ciclo celular e impedindo a replicação do DNA danificado (Vermeulen *et al.,* 2003; Herranz *et al.,* 2018). Estresse persistente pode ativar $p16^{INK4A/Rb}$ um supressor de tumor codificado pelo *locus* ARF-INK4 (gene *CDKN2A, CDKN2B*), que também codifica $p19^{ARF}$ ($p14^{ARF}$ em humanos e $p19^{ARF}$ em camundongo); enquanto $p16^{INK4A}$ inibe seletivamente CDK4 e CDK6 (ciclinas essencial para a entrada da célula em G1, fase de preparação para a divisão celular), $p19^{ARF}$ previne a degradação de p53(Vermeulen et al., 2003; Herranz et al., 2018). Em galinhas (*Gallus gallus*), embora a degradação do gene *TP53* p53 seja inibida por uma versão truncada da proteína ARF, não há uma versão que codifique a proteína INK4a. A parada do ciclo celular mediada pela ligação às CDKs 4 e 6 é executada pela proteína $p15^{INK4b}$, codificada pelo gene *CDKN2B* (Kim *et al.,*2003).

Além de sair do ciclo celular, as células senescentes produzem e secretam uma combinação complexa de fatores, coletivamente referidos como o Fenótipo Secretor Associado à Senescência (SASP, do inglês *Senescence-Associated Secretory Phenotype*). SASP é o termo geral dado à combinação de citocinas, quimiocinas pró-inflamatórias, proteases de matriz extracelular, fatores de crescimento, vesículas extracelulares e outras moléculas sinalizadoras secretadas por células senescentes e acredita-se que sua composição específica varia dependendo do tipo de célula e do indutor da senescência (Herranz *et al.,* 2018). As funções atribuídas ao SASP são muito diversas e dependem não apenas da natureza do SASP, mas do ambiente circundante e do contexto genético das células expostas ao secretoma senescente (Herranz *et al.,* 2018). Embora os eventos que ativam o SASP ainda sejam pouco compreendidos, eles normalmente estão conectados à DDR (Birch *et al.*, 2020). A atividade persistente de DDR é necessária para a indução de vários fatores pró-inflamatórios SASP, como Interleucina (IL)-6 e IL-8 (Birch *et al.,* 2020). A expressão de IL-1b aumenta em resposta a infecção bacteriana, viral e parasitária, em acordo com seu papel de mediador pró-inflamatório (Gibson *et al.,* 2014). O SASP possui alguns efeitos benéficos, permitindo o recrutamento do sistema imunológico para lesões pré-malignas e promoção do reparo de tecidos danificados; porém, a secreção de muitos fatores pró-inflamatórios, como IL-6, IL-8, proteínas cofatores de membrana (MCPs), proteínas inflamatorias de macrófagos

(MIPs), proteínas e enzimas implicadas na reorganização da matriz extracelular (MEC) como as Metaloproteinases de Matriz (MMPs), podem levar a efeitos deletérios como promoção da proliferação e inflamação, tanto de forma autócrina quanto parácrina (Lopes-Paciencia *et al.,* 2019). Além disso, a quantidade de moléculas liberadas pelas células senescentes e a duração da liberação do SASP parece ser importante para levar aos efeitos deletérios causados pelas células senescentes (Paramos-de-Carvalho *et al.,* 2021).

Sabe-se que a infecção bacteriana e viral pode induzir senescência das células hospedeiras já que, infecção viral desencadeia a ativação de muitas vias celulares de resposta ao estresse, como choque térmico, estresse oxidativo ou DDR (Seoane *et al.,* 2020). Entretanto, até agora, os papéis de bactérias e vírus na indução da senescência celular ainda são amplamente desconhecidos. A senescência pode contribuir para controlar a replicação viral ou promover a produção de vírus, como por exemplo, a característica de parada do ciclo celular estável que define a senescência celular pode beneficiar alguns agentes virais como o Vírus da Imunodeficiência Humana (HIV) que é mais transcricionalmente ativo na fase G2 do ciclo celular. Porém ainda são necessários estudos para compreender esta associação entre infecções virais e senescência celular (Seoane *et al.,* 2020).

A indução inoportuna da senescência está ligada a muitas doenças adultas e está presente durante o desenvolvimento embrionário, estudos mostram que durante a embriogênese, a senescência é detectada principalmente na crista ectodérmica apical dos membros e na placa do teto do tubo neural, curiosamente, essas estão entre as áreas frequentemente afetadas por anomalias congênitas induzidas por teratógenos (Storer *et al.*, 2013). Como apresentado anteriormente, as células senescentes são determinadas por várias características, incluindo a DDR, atividade de β-galactosidase (SA-β-gal), expressão aumentada de inibidores de CDK e secreção de moléculas específicas (Wei *et al.,* 2018). Recentemente, um estudo relacionou alterações em marcadores de senescência celular com a teratogênese induzida por ácido valpróico (VPA), um fármaco anticonvulsivante relacionado a anomalias do sistema nervoso (Rhinn *et al.,* 2022). O estudo de Rhinn et al. (2022) expôs que p21, p19 e p16 e os genes SASP*, IL6*, *IL1a*, *IL1b* e *Pai1* apresentaram sua expressão alterada em embriões de camundongo expostos a VPA, indicando que a exposição à este fármaco causa uma indução pronunciada de senescência em células neuroepiteliais, levando a uma diminuição acentuada na proliferação e neurogênese. Estes achados, indicam que essa via celular pode estar relacionada com a ocorrência de microcefalia, particularmente a induzida por agentes teratogênicos e que a utilização destes marcadores na identificação de presença de

células senescentes auxilia na compreensão de suas funções biológicas em estados normais e patológicos.

1.3 EMBRIÃO DE GALINHA

A combinação de modelos animais e pesquisas sobre senescência celular não apenas amplia nossa compreensão dos mecanismos envolvidos, mas também abre novas perspectivas para o desenvolvimento de intervenções terapêuticas e estratégias de prevenção em condições relacionadas à senescência, sendo possível desenhar estratégias e solucionar problemas de saúde pública em nossa sociedade. Os modelos animais são capazes de fornecerem informações significativas para compreensão de doenças, e têm sido muito utilizados para desenvolvimentos de vacinas e compostos farmacológicos pois possibilitam experimentos para avaliar potenciais intervenções terapêuticas, contribuindo para a identificação de estratégias preventivas e terapêuticas direcionadas. No caso de condições como a SCZ, que ocorre devido a um processo de teratogênese, o uso de modelos animais para compreender seus mecanismos é essencial.

Atualmente, uma ampla gama de modelos animais é empregada em estudos de teratogênese experimental. A seleção cuidadosa do modelo mais apropriado para o experimento desempenha um papel crucial na obtenção de resultados robustos e significativos (Wachholz *et al.*, 2021a). No caso de ZIKV, o embrião de galinha é um modelo relevante, pois é naturalmente suscetível à infecção pelo vírus e frequentemente usado como sentinela para detecção de arboviroses (Wachholz *et al.*, 2021a; Thawani *et al.,* 2018). Devido à facilidade de manuseio e infecção, este modelo animal tem sido utilizado em diversos estudos neste tema (Goodfellow et al., 2016; Thawani *et al.*, 2018; Wachholz *et al.,* 2021b).

A galinha (*Gallus gallus*) é um dos animais domésticos mais comuns no mundo e seu ovo é uma fonte tão acessível de embriões que atraiu o interesse dos antigos egípcios (Robinson et al., 2019). O uso desse modelo animal para compreender o desenvolvimento embrionário ultrapassou os séculos devido a sua fácil manipulação, baixo custo de obtenção e manutenção, além de fácil visualização do embrião, já que este é grande e se encontra fora do corpo materno. Outro ponto importante deste modelo é o estagiamento de desenvolvimento embrionário bem documentado (Hamburguer e Hamilton, 1951), o que permite acompanhar a formação do embrião em diversos momentos e usar como comparativo quando necessário.

1.4 JUSTIFICATIVA

Nas últimas décadas, pudemos observar episódios cada vez mais frequentes de surtos, epidemias e pandemias ocasionadas por vírus ao redor do mundo, que são facilitados pela globalização e aquecimento global. Emergências em saúde como essas não apenas custam vidas, mas também economias globais, afetando não somente os acometidos diretamente com a doença, mas toda a população. As consequências de doenças causadas por infecção viral como a Síndrome Congênita do Zika, marcada pela ocorrência de microcefalia, são sérias e permanentes. Crianças acometidas por essa síndrome, quando na forma agressiva, necessitam de cuidados diários e especiais e as sequelas gerais da infecção ainda não são bem estabelecidas, vide que o surto por ZIKV é recente e ainda é desconhecido o desenvolvimento comportamental e cognitivo das crianças.

Estudos que entendam e elucidem os mecanismos causadores de SCZ são de extrema importância. Além do aumento na compreensão dos fatores envolvidos na ocorrência da microcefalia, da relação entre senescência celular e desenvolvimento do sistema nervoso, será através do entendimento dos mecanismos relacionados à infecção pelo ZIKV e a ocorrência da SCZ que poderemos, potencialmente, desenvolver estratégia preventivas dos danos causados pelo ZIKV no desenvolvimento embrio-fetal.

Os modelos animais desempenham um papel crucial nesse cenário, oferecendo uma forma valiosa para investigar a fisiologia, patologia e desenvolvimento de doenças No contexto do ZIKV, o embrião de galinha emerge como um modelo pertinente, uma vez que demonstra natural suscetibilidade à infecção e é de fácil acesso e manuseio, fornecendo um ambiente controlado para estudar os eventos biológicos relevantes, mas também possibilitam a manipulação experimental.

Recentemente, foi observado que a senescência celular é um dos mecanismos relacionados à anomalias do sistema nervoso causadas por agente teratogênico. Pouco se sabe dos mecanismos moleculares relacionados à teratogênese do ZIKV e com base nas recentes observações, se torna necessário caracterizar os mecanismos e funções das células senescentes no contexto de infecção pelo vírus Zika tendo em vista que a microcefalia é a principal característica da SCZ. Portanto, a integração de modelos animais na pesquisa sobre SCZ não apenas enriquece nosso entendimento da patogênese, mas também abre caminho para a formulação de estratégias preventivas.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar os níveis de expressão de marcadores de senescência celular na teratogênese do ZIKV, utilizando embriões de galinha como modelo experimental.

2.2 OBJETIVOS ESPECÍFICOS

a. Análisar a expressão dos genes *CDKN2A (*ARF*), CDKN1A (*p21*), CDKN2B* (p15INK4b*), TP53 (*p53*), IL1B (*IL-1b*), IL6 (*IL-6*)* e *MMP9 (*MMP 9*),* envolvidos com a senescência celular e fenótipo secretor associado a senescência em amostras de embriões de galinha expostos e não expostos ao ZIKV.

b. Comparar os dados de expressão gênica entre os grupos de embriões expostos e não expostos ao Zika Vírus.

3. ARTIGO CIENTÍFICO

Expression analysis of Cellular Senescence-related genes in a chicken embryo model of ZIKV-induced Microcephaly

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Abstract: Zika virus (ZIKV) was discovered in Uganda in 1947 and gained global attention with a significant outbreak in Brazil in 2015, leading to a sharp increase of microcephaly cases in newborns of infected mothers. From this, ZIKV was established as a new human teratogen, causative agent of the Congenital Zika Syndrome (CZS). So far, mechanisms that lead to CZS are not fully understood and disruptions on cell cycle have been linked to its occurrence. Cellular senescence, marked by cell cycle arrest and the Senescence-Associated Secretory Phenotype (SASP), is implicated in congenital anomalies induced by teratogenic agents. Here, we evaluated the expression of genes related to cellular senescence in chicken embryos exposed to ZIKV and controls. For this, 50 μL of culture medium or culture medium containing 3x10^4 PFU of ZIKV was applied to embryos at the HH11-12 stage, incubated for 24 or 96 hours. PCR was performed using cDNA obtained from the embryos' brain. The expression of genes CDKN2A, CDKN1A, CDKN2B, TP53, IL1B, IL6, and MMP9 was tested; basal expression of some of the genes was not obtained. Despite *CDKN1A* (p21) and *MMP9* genes showing subtle variations, statistical significance was not found.The complexity of these mechanisms underscores the need for additional investigations to understand the relationship between senescence and ZIKV teratogenesis. Further research, including analysis of additional SASP markers could shed light on the relationship among ZIKV, senescence, and microcephaly.

Keywords: embryo, genes expression, virus infection, congenital defects, mesencephalon.

1. Introduction

Zika virus (ZIKV) belongs to the positive-strand RNA family Flaviviridae. Its transmission occurs through the *Aedes aegypti* mosquito, representing all flavivirus vectors (Schuler- Faccini et al., 2016). ZIKV was first identified in Rhesus monkeys in Uganda in 1947, and for decades, only sporadic cases were recorded in Africa and Asia (Giraldo et al., 2023). In the 21st century, initial reports of epidemics emerged, highlighting a significant outbreak in Brazil in 2015, associated with an increase in cases of microcephaly in babies of infected mothers, differing from other countries with low-impact and non-lethal infections (Schuler- Faccini et al., 2016; Cugola et al., 2016). The anomalies observed in babies exposed to ZIKV during embryonic and/or fetal development are part of Congenital Zika Syndrome (CZS), marked mainly by the occurrence of microcephaly.

Knowledge about SCZ natural evolution, associated risk factors and causative mechanisms is important. Previous studies have found that ZIKV is neurotropic and able to infect and disrupt the cell cycle (Garcez et al., 2016). Moreover, SCZ has been linked to increased expression of genes associated with the inflammatory response, increased expression of genes involved in apoptosis (Lima et al., 2019). In a recent study, cellular senescence was pointed as one of the mechanisms related to congenital anomalies caused by valproic acid, a teratogenic drug that causes birth defects, cognitive impairment, and autism spectrum disorder (Rhinn et al., 2022). In this study, it was found a pronounced induction of senescence in neuroepithelial cells associated with a marked decrease in proliferation and neurogenesis (Rhinn et al., 2022). It is known that viral infection triggers the activation of many cellular stress response pathways, such as oxidative stress or DNA damage response (DDR) (Seoane et al., 2020). Senescent cells remain metabolically active but do not respond to mitogenic stimuli, undergoing significant changes in chromatin, gene expression and morphology (Ben-Porath & Weinberg, 2005; Chen et al., 2006). Tumor suppressor genes play a crucial role in this process by influencing the cell cycle checkpoint machinery (Chen et al., 2006).

The transition between cell cycle phases is regulated by proteins such as cyclin-dependent kinases (CDK). In senescence, cycle arrest is induced by damage and depends on TP53, which stimulates the transcription of p21, this, in turn, inhibits CDK, resulting in cell cycle arrest and prolonged exposure to stress or additional stimuli can trigger the activation of $p16^{INK4a}$, an inhibitor of cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), resulting in a prolonged cell cycle arrest (Herranz & Gil, 2018). Senescent cells

produce and release a complex mixture of factors known as the Senescence-Associated Secretory Phenotype (SASP). The SASP includes cytokines, pro-inflammatory chemokines, extracellular matrix proteases, growth factors, and other signalling molecules (Herranz & Gil, 2018). SASP varies depending on the cell type and senescence inducer but is mainly a combination of cytokines, chemokines, extracellular matrix proteases, growth factors and matrix metalloproteinases (MMPs and other signalling molecules secreted by senescent cells) (Coppé et al., 2010; Herranz & Gil, 2018). MMPs, such as MMP-9, regulate inflammatory functions (Rojas-Quintero et al. 2018).

The specific roles of bacteria and viruses in inducing senescence are still poorly understood. A profound understanding of biological processes leading to microcephaly and other congenital defects caused by CZS is essential to design strategies for addressing societal and public health issues. Animal models are capable of providing significant information for understanding diseases. In the context of ZIKV, the chicken embryo is a crucial model because, unlike rodents, it is naturally susceptible to virus infection and is often used as a sentinel for the detection of arboviruses (Thawani et al., 2018; Wachholz et al., 2021a) Therefore, this study aims to evaluate molecular markers of cellular senescence using chicken embryos exposed to ZIKV as a model.

2. Methods

This study followed the recommendations of the ARRIVE Guidelines (Kilkenny et al, 2010) and used biological samples from a project previously approved by the Ethics Committee on Animal Use (CEUA) of Universidade Federal do Rio Grande do Sul (UFRGS) registered number 35508. The use of samples was approved by the CEUA of Hospital de Clínicas de Porto Alegre (HCPA; registered number 2020-0209). All animal experiments were performed according to the law 11.794/2008.

2.1. Experimental Groups

Four experimental groups of animals were used to compare molecular markers of cellular senescence: embryos infected with ZIKV ($3x10^4$ ZIKV Plaque Forming Units) and control embryos (treated with culture medium), evaluated at two different moments, being 24 hours post-infection (hpi) and 96 hpi. For the phenotypic evaluation, the evaluators were blinded to the groups.

2.2. Embryology and Viral Inoculation

The ZIKV strain 17, isolated in Brazil from a 33-year-old man in 2016 (Oliveira et al., 2018) was multiplied in mammalian cells (E6 Vero, ATCC CRL-1586), maintained in a specific culture medium and preserved at -80°C until use. Fertilized non-transgenic chicken eggs of the Cobb-Vantress strain were transferred to an incubator at a temperature of 37°C and humidity of 60% and maintained until the embryos reached the HH 10-12 (E1.5) developmental stage (Hamburger & Hamilton, 1951). A small hole was made in the egg to manipulate the embryo. Then, a small window was opened in the eggs, and 50 µL of culture medium or culture medium containing $3x10^4$ PFU of ZIKV or culture medium only was applied over the embryo. After manipulation, the window was closed with adhesive tape, and the embryos returned to the incubator. The embryos remained incubated for 24 or 96 hours and after that, they were euthanized and heads were photographed (embryos kept for 96 hpi) and further processed for RNA extraction. Malformations and head size was evaluated in the embryos that remained for 96 hpi to confirm the malformation phenotype. Mesencephalon vesicle was measured to observe the presence of a smaller area of the developing brain, corroborating the finding of microcephaly. Embryos of 24 hours were not photographed due to their small size. Samples were stored at -80°C until RNA extraction.

2.3. Molecular Analysis

Total RNA was extracted from brain embryos using PureLinkTM RNA Mini Kit (Invitrogen) and cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). The genes evaluated by quantitative PCR (qPCR) were *CDKN1A (*p21*), MMP9, TP53 (*p53*), CDKN2A (*ARF*), CDKN1B (*$p15^{INK4b}$*), IL1B (*IL-1b*)* and *IL6 (*IL-6*)* and the *ACTA1* gene was used as a normalising gene. Such reactions were performed using specific primers (Table 1) to amplify coding regions of the genes. The total sample sizes are indicated in Figure 1c legend.

2.4. Imaging and Data Analysis

Embryo brain morphometry was performed using Image J software (National Institute of Health, USA), and mesencephalon measured. Data analysis and representation were conducted using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). The analysis of relative gene expression used the calculation using the $2^{-\triangle\triangle Ct}$ method . Comparison of gene expression between ZIKV and control groups at two different time-points, 24 hpi and 96 hpi

was conducted using a generalized linear model with gamma distribution and logarithmic link function. For all statistical tests, a p value <0.05 was considered statistically significant.

3. Results

In embryos exposed to ZIKV, we observed the morphological abnormalities in the developing brain, here, we found that embryos exposed to ZIKV presented encephalic malformations (Figure 1a) and a significant reduction in the midbrain area when compared to controls (Figure 1b). The phenotype resembled the smaller head, what is observed in humans (Figure 1a) and is according to a previous work from our group (Wachholz et al., 2021b).

We were able to investigate the expression only of *CDKN1A* and *MMP9* genes at the tested time-points since basal expression of *TP53, CDKN2A, CDKN1B, IL1B* and *IL6* genes was not detected. Therefore, we verified the expression only of *CDKN1A* and *MMP9* genes in chicken embryos heads exposed to ZIKV and compared them to controls in two different time-points. In the analysis of *CDKN1A* gene expression, no difference in expression patterns was detected between the groups ($p < 0.920$), regardless of the exposure time-point. However, at 96 hpi, a slight disparity in expression between the two treatments was identified; however, this difference did not reach significance (Figure 1c). In the case of *MMP9* (Figure 1c), the data revealed a certain equality in the gene expression profiles between ZIKV and controls, both at 24 and 96 hpi ($p<0.942$), suggesting an absence of distinctive expression responses of this gene in embryos infected with the virus.

4. Discussion

ZIKV is a teratogenic agent transmitted through mosquito bites, capable of causing congenital anomalies in children exposed to the infection during embryonic development, thus being a global health problem given that its transmitter, the mosquito, is cosmopolitan. The anomalies caused by ZIKV are mainly associated with the central nervous system, and make up the CSZ, in which one of the main characteristics is microcephaly (Wachholz et al., 2021b). Cellular senescence plays a relevant role in neurodevelopmental defects caused by exposure to a teratogen, and it is known that inadequate or chronic induction of senescence contributes to ageing and the emergence of diseases (Rhinn et al., 2022). Despite this, the exact molecular pathways responsible for brain abnormalities caused by ZIKV viral infection

is still unknown. Here, we analysed the possible role of cellular senescence in CZS using chicken embryos as experimental models. We found that many of the genes we tested did not present a basal expression, at least during the analysed embryonic period. For those that showed basal expression, subtle variations were observed, and no statistical significance was found between control and ZIKV-exposed embryos.

Cellular senescence can be categorised as a natural biological process during embryonic development or a process induced by prolonged exposure to stress, which generates cellular and tissue changes (Lee et al., 2023). Cells exhibit a stable cell cycle arrest when they become senescent, and this process is regulated by gene pathways, one of which is increased expression of *TP53*, which upregulates *CDKN1A* and arrests the cell cycle (Lee et al., 2023). In addition, the SASP is a crucial feature of cellular senescence. It is characterised by exocytosis of pro-inflammatory cytokines that can potentially influence neighbouring, metabolically normal cells to become senescent (Childs et al., 2014). Senescent cells can be eliminated by immune cells attracted by SASP pro-inflammatory factors. However, MMPs secreted by senescent cells cleave immune cell surface proteins, preventing their cell death and resisting apoptotic death, leading to persistent senescent signals which can be fatal to adjacent cells (Lee et al., 2023). MMP9 cleaves several proteins to regulate inflammatory and injury responses (Rojas-Quintero et al., 2018).

By evaluating molecular markers of cellular senescence in chicken embryos exposed to ZIKV, we could observe that *CDKN1A* and *MMP9* genes did not show a difference in expression between control and ZIKV at the tested time-points. As demonstrated, a subtle discrepancy in the expression of the *CDKN1A* gene was observed between the ZIKV and controls particularly at 96 hours post-infection, when a higher expression occurred in embryos exposed to ZIKV (Figure 1c). These subtle variations might have biological relevance, indicating a slight increase in the expression of this gene. This, in turn, could interrupt cell replication, preventing midbrain cells from multiplying and potentially contributing to the development of microcephaly. It is crucial to highlight that senescence is a standard developmental mechanism during embryogenesis (Storer et al., 2013). However, the lack of statistical significance can be attributed to data variability without excluding the observations' biological importance.

It is imperative to persist in investigations related to senescence and microcephaly. Senescence represents a mechanism of notable interest and is recognised as a typical response

to viral infections in the respiratory system (Kelley et al., 2020). It is positioning itself as a known protective response of the body in the face of infections. Continuing investigations exploring different senescence markers, such as other SASP molecules and β-galactosidase (SA-β-gal), may be a promising approach. A combination of markers can be used to identify senescent cells, given that none of the markers is singular or specific for cellular senescence. Therefore, more studies are needed to elucidate these gaps in knowledge about ZIKV and the processes that cause SCZ.

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Table 1. Primers and PCR amplification efficiency of candidate reference gene.

Gene	Primer sequence	Produce size	R^2	slope	E (100%)
CDKN1A	F1: GGGCAGACCACCATCAAAGA R1: ACTCGGCATTGCTTCGTACA R2: GTACAGGGCAGGGAAAGAC	174	0.991	-3.544	91.517
CDKN2B*	F1: CCCAACGCRGTCAACTCCTT R1:AGCGTGTCCAGGAAGCC F2: AACTCCTTCGGTCGGACCC	-	-	-	-
CDKN2A*	F5.1: GGGTTCTGAGAAGGAGGCAC R5:GCCAGCGTGACCAGGAAG F5.2: GTTCTGAGAAGGAGGCACCG R5:GCCAGCGTGACCAGGAAG	-	-	-	-
MMP9	F7: GGATGGGATGCTGTGGTGTG R7: TGAAGGGGAAGACGCAGG F8: TCACCCAGATTTACAGCGGC R8: CATCGCCCTGAATCCCACTG	128	0.996	-3.526	92.153
TP53*	F8: ACCACCACCTCTGCCATTG R8: AAGTCCCCCCCATAATCCTCCGT F7: ATCCTCACCATCCTTACACTGG R7: TTCGGTTGGGGGCGACAT	- - - -	- - - -	- - - -	- - - -
ACTA	F:: GTACCACCATGTACCCAGG R: : CAAGATGGAGCCACCGAT	134	0.996	-3.192	105.78
IL1B*	F: ACTGGGCATCAAGGGCTA R:GGTAGAAGATGAAGCGGGTC	-	-	-	-
IL6*	F: TTTATGGAGAAGACCGTGAGG R:TGTGGCAGATTGGTAACAGAG	-	-	-	-

Note: Amplification efficiency will affect the accuracy of experimental data. Theoretically, the optimal value is 100%, indicating that the template is copied exponentially, and it is controlled between 90% and 110%, which is the accepted error range nowadays [9].

*: not possible to obtain basal expression.

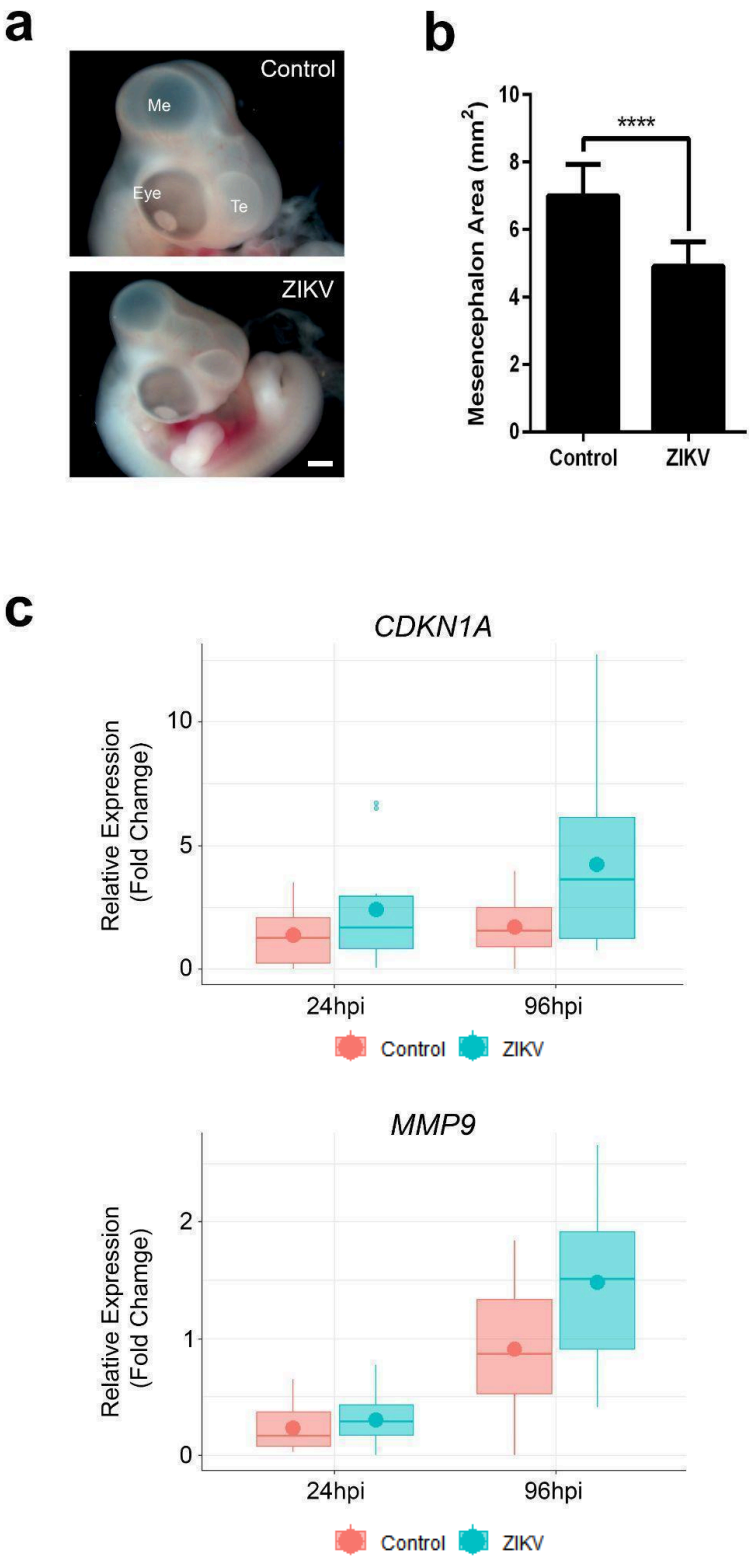


Figure 1: *Zika virus affects chicken embryo development and does not influence expression of senescence-related genes. (A) The presence of the Zika virus (ZIKV) during the HH 10-12 stage results in an impact on brain growth and causes brain defects. (B) Midbrain area is reduced in ZIKV-infected embryos compared to controls after 96hpi (C) Box plot of relative gene expression indices for controls and ZIKV-infected at two time-points, in the sample size of 37 for CDKN1A (9 control 24hpi, 9 control 96hpi, 11 ZIKV 24hpi, 8 ZIKV 96hpi), and 38 samples for MMP9 (10 control 24hpi, 10 control 96hpi, 9 ZIKV 24hpi, 9 ZIKV 96hpi).*

4. CONCLUSÕES E PERSPECTIVAS

O ZIKV é um agente teratogênico que, através da transmissão por picada de mosquito em gestantes, provoca anomalias congênitas nos embriões/fetos em desenvolvimento, principalmente no sistema nervoso central, resultando na SCZ, notadamente caracterizada pela microcefalia. Este estudo buscou entender a relação entre a senescência celular e os defeitos de neurodesenvolvimento causada pela exposição ao ZIKV, através de marcadores moleculares de senescência.

A senescência celular é um processo normal durante o desenvolvimento embrionário, pode ser causada por estresses prolongados, influenciando o ciclo celular através da regulação gênica, como p53 e p21. Este processo afeta o metabolismo celular, resultando em SASP, marcados pela exocitose de citocinas pró-inflamatórias e metaloproteinases de matriz (MMPs), como a *MMP-9*. Avaliando embriões de galinha expostos ao ZIKV e controles, observou-se que genes relacionados à senescência (*CDKN1A e MMP9*) não apresentaram diferença significativa de expressão entre grupos.

Ampliar o tamanho amostral pode fornecer uma detecção mais precisa das expressões gênicas. A variabilidade genética e a ativação de diferentes vias de senescência em resposta à infecção viral são fatores a serem considerados. Embora os resultados não tenham sido conclusivos, a persistência na investigação da relação entre senescência e microcefalia no contexto do ZIKV é crucial, preenchendo assim lacunas no conhecimento desses processos. A exploração de diferentes marcadores de SASP pode ser uma abordagem valiosa para estudos futuros.

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA

AUTHOR GUIDELINES

GUIDELINE SECTIONS

1. Submission
2. Aims and Scope
3. Manuscript Categories and Requirements
4. Preparing the Submission
5. Editorial Policies and Ethical Considerations
6. Author Licensing
7. Publication Process After Acceptance
8. Post-Publication
9. Editorial O ce Contact Details

1. SUBMISSION

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2. AIMS AND SCOPE

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The journal ***Birth Defects Research*** publishes original research and reviews as well as short communications, case reports, opinions and practice and policy articles in areas related to the discovery, description, etiology, and prevention of adverse reproductive and developmental outcomes. In particular the journal is devoted to the publication of original research that contributes to the understanding of prenatal development and the causes and mechanisms leading to adverse pregnancy outcomes including pregnancy loss, structural and functional birth defects, and e ects on maternal and children's health, and to the identi cation of biological mechanisms and interventions that reduce these risks.

Adverse reproductive and developmental outcomes may have genetic, environmental, nutritional or epigenetic causes. Accordingly, the journal ***Birth Defects Research*** takes an integrated, multidisciplinary approach in its organization and publication strategy.

The journal ***Birth Defects Research*** publishes original research and reviews in the following areas:

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Cellular and molecular mechanisms of adverse developmental and reproductive outcomes.
Protective factors and their mechanisms
Reproductive and developmental toxicity of drugs, chemicals and biotechnology-derived products, and organismal, cellular and computational approaches to detecting and understanding toxicity to prevent birth defects and other adverse reproductive outcomes.
physiologically based pharmacokinetic (PBPK) mathematical modeling of pregnancy and delivery of agents to the developing organism;
approaches to risk assessment for developmental and reproductive toxicants;
toxicity related to direct exposure in juvenile animals
Parental nutritional, behavioral and socioeconomic factors that positively or negatively a ect the risk of adverse reproductive outcomes.

Birth Defects Research is published in twenty issues per year.

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Clinical and Molecular Teratology
Developmental and Reproductive Toxicology
Embryo Today: Reviews (editor-invited submissions)*

*Please contact Michiko Watanabe (**mxw13@case.edu**) regarding invited papers on the subject of "Embryo Today: Reviews".

Manuscript Types

Research Article: Manuscripts describing the results of original research conducted in the broad areas of reproduction, birth defects, other adverse pregnancy outcomes, and maternal and children's health. Research articles should include: Abstract, Introduction, Methods, Results, and Discussion. The abstract should be structured as follows: Background, Methods, Results, and Conclusions (250-word limit).

Review Article: Review articles focusing on topics of broad scienti c interest to investigators in the areas listed above for research articles. Review articles are usually solicited by the Editors; We encourage you to contact an Editor if you wish to propose a Review Article.

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Case Report: Articles describing one or more cases of a unique or rare clinical nding or syndrome. The Abstract for a Case Report should be structured as Background, Case(s), and Conclusions. The text should use the following format: Introduction, Case Report, and Discussion. The Discussion is expected to place the subject(s) of the Case Report in the context of the published literature on the same or related conditions or birth defects and to discuss the possible pathogenesis of malformations.

Opinion: Articles presenting new data that lead to a speci c hypothesis warranting further research (see and **114 (20): 1313-1323, 2022** for examples). The format of Opinion articles should follow those outlined for Research Articles.

Practice and Policy: These articles set forth guidelines related to the understanding and prevention of birth defects (see **113 (3): 218-226, 2021** for an example). Guidelines should be formatted with the same organization as Research Articles.

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4. PREPARING THE SUBMISSION

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Book

Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

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