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ALTERAÇÕES METABÓLICAS INDUZIDAS POR ZIKA VÍRUS ATENUADO EM CÉLULAS DE GLIOBLASTOMA.

METABOLIC ALTERATIONS INDUCED BY ATTENUATED ZIKA VIRUS IN GLIOBLASTOMA CELLS.

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Dedico esse trabalho a minha família e namorada, por toda paciência, amor e investimento em mim depositados.

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RESUMO

O Zika vírus (ZIKV) tornou-se recentemente um grande motivo de preocupação, uma vez que sua associação com casos de microcefalia em recém-nascidos começou a ser investigada e confirmada. A partir de então, o ZIKV tem sido intensamente estudado e vários dados científicos demonstram o tropismo do vírus por células neurais. Estudos prévios propuseram que o ZIKV induz morte de células de glioblastoma, sugerindo que as moléculas associadas ao ZIKV e o próprio ZIKV seriam capaz de induzir alterações bioquímicas intracelulares e, assim, ser alternativas para o controle do câncer neural. Nesse sentido, o presente trabalho apresenta uma abordagem prospectiva para o manejo do glioblastoma: produzir uma terapia baseada no ZIKV que estimule o controle do glioblastoma. Desenvolvemos um protótipo de Zíka vírus atenuado baseado em vesículas de membrana externa bacteriana (ZVp). O ZIKV atenuado foi aplicado nas células de glioblastoma, onde os efeitos citopáticos e citostáticos foram avaliados. Células de glioblastoma, com e sem tratamento, foram submetidas à análise por espectrometria de massa. Resultados: A análise microscópica mostrou efeitos citopáticos induzidos pelo protótipo ZIKV nas células de glioblastoma após 24 e 48 horas pós-tratamento. Ensaio de fragmentação de DNA e expressão de TNF-alfa foram indicativos de que ZVp induziu dano celular e morte. A investigação metabolômica elegeu 5 diferentes biomarcadores que podem estar associados aos efeitos citopáticos celulares, destacando as modificações bioquímicas intracelulares induzidas pelo ZIKV atenuado. A evidência notável de morte celular no glioblastoma estimulou estudos adicionais que renderam uma triagem preliminar com outras linhagens de células tumorais. Entre os 11 tumores avaliados, os tumores de próstata e ovário foram os mais afetados pelo protótipo ZIKV, e outras 6 linhagens celulares também apresentaram efeitos citostáticos. Conclusões: Os resultados demonstraram que este protótipo não apenas surge como uma possível alternativa para o controle do glioblastoma, mas também pode ser uma ferramenta importante contra outros tumores importantes.

PALAVRAS CHAVE: Zika vírus; Vesícula de membrana externa; Protótipo; Espectrometria de massa; Glioblastoma.

ABSTRACT

Zika virus (ZIKV) has recently become a major matter of concern since its association with microcephaly cases in newborns was determined. From then on, ZIKV has been untiringly studied, and several scientific data have shown the virus' tropism to neural cells. Previous studies have proposed that ZIKV induced glioblastoma cells death, suggesting that ZIKV and ZIKV-associated molecules might induce intracellular biochemical alterations, and thereby be alternatives for neural cancer management. In this sense, the present contribution presents a prospective approach for glioblastomas management: producing a ZIKV-based therapy that stimulates glioblastoma control. We developed an attenuated ZIKV prototype based on bacterial outer membrane vesicles (OMV). The attenuated ZIKV was applied on glioblastoma cells, where cytopathic and cytostatic effects were evaluated. Glioblastoma cells, with and without treatment, were submitted to mass spectrometry analysis. Results: Microscopic analysis showed cytopathic effects induced by ZIKV prototype on glioblastoma cells after 24 and 48 hours post-treatment. DNA fragmentation assay and TNF-alpha expression were indicative that ZVp induced cell damage and death. The metabolomics investigation elected 5 different biomarkers that might be associated with cell cytopathic effects, highlighting intracellular biochemical modifications induced by the attenuated ZIKV. The remarkable evidence of cell death in glioblastoma stimulated further studies that rendered a preliminary screening with other tumor cell lineages, though anti-proliferative activity test. Among the 11 tumors evaluated, prostate and ovarian tumors were the most affected by ZIKV prototype, and other 6 cell lines also presented cytostatic effects. Conclusions: Results ultimately demonstrated that this prototype not only emerges as a potential alternative for glioblastoma management, but may also be an important tool against other important tumors.

KEYWORDS: Zika virus; Outer membrane vesicle; Prototype; Mass spectrometry; Glioblastoma.

LISTA DE ABREVIATURAS E SIGLAS

CT:	*Control group*
ESI-HRMS:	*Electrospray ionization and high-resolution mass spectrometer*
GBM:	*Glioblastoma*
hNPCs:	*Human Neural Progenitor Cells*
hNSCs:	*Human Neural Stem Cells*
MS:	*Methionine synthase*
m/z:	*mass / ion charge*
NS:	*Non-structural protein*
OMV:	*Outer membrane vesicles*
PLS-DA:	*Partial least squares discriminant analysis*
SI:	*Selectivity index*
VIP score:	*Variable Importance in Projection score*
ZIKV:	*Zika virus*
ZVp:	*Zika virus prototype*

SUMÁRIO

Introdução

Zika vírus

O Zika vírus (ZIKV) é um arbovírus (Arthropod-borne virus) transmitindo basicamente por duas espécies de mosquitos, *Aedes aegypti* e *Aedes albopictus*, sendo o primeiro o principal vetor de transmissão (1). Além da transmissão pelos mosquitos, o Zika vírus pode ser ativamente transmitido entre humanos por relações sexuais desprotegidas, transfusão de sangue, relação materno-fetal e até mesmo pelo contato com fluidos corporais, tais como: urina, lágrimas e suor (2). O Zika vírus recebeu essa nomenclatura, pois foi encontrado e caracterizado pela primeira vez na floresta de Zika localizada na Unganda em 1947 (1). Nessa época, macacos da espécie *Rhesus* eram levados até essa floresta em pequenas gaiolas e permaneciam enjaulados em locais estratégicos entre 7 e 15 dias. Após esse tempo, os pesquisadores regressavam aos locais determinados e retornavam aos centros de pesquisas com os macacos sobreviventes. Então, eram realizados testes sanguíneos para a identificação de novas linhagens de vírus e bactérias. Desta maneira, a primeira linhagem de Zika vírus foi reportada (3). O ZIKV pertence à família *Flaviviridae* e gênero dos *Flavivirus*, sendo geneticamente próximo aos vírus da Dengue (DENV) e vírus da febre amarela (YFV). Os *Flavivirus* são caracterizados por conterem uma única fita de sentido positivo de RNA e possuírem um diâmetro entre 20 e 30 nanômetros (4). Assim como os outros *flavivirus*, o ZIKV adentra as células por endocitose e são conduzidos pela interação do seu envelope proteico e os receptores das células hospedeiras. O genoma viral é liberado no citoplasma celular onde será traduzido, iniciando-se um possível efeito citopático (5). Os principais sintomas clínicos dados pela infecção por Zika são: febre, lesões na pele, dores de cabeça, dores nas articulações, conjuntivite e dores musculares. Além disso, a infecção por Zika vírus pode levar ao desenvolvimento da síndrome de Guillain-Barré em adultos e condições de microcefalia em bebês neonatos. As metodologias existentes atualmente para o diagnóstico laboratorial consistem no exame sorológico do paciente, buscando a detecção de anticorpos IgM por testes imunocitoquímico

e no exame virológico que consiste na utilização de técnicas de Reação em Cadeia da Polimerase de Transcriptase (RT-PCR) (1).

O primeiro surto de infecção por ZIKV foi relatado na Polinésia Francesa entre 2010 e 2013. Nesse período, algumas alterações neurológicas em recém-nascidos de mulheres infectadas foram observadas, assim como complicações neurológicas em adultos. Entretanto, até essa época essas alterações não foram comprovadamente correlacionadas com a infecção por ZIKV. Entre 2013 e 2014 o Zika vírus chegou ao Brasil e provocou a maior epidemia causada por ZIKV já registrada. Uma das suspeitas é que o vírus foi trazido e espalhado principalmente por visitantes da Polinésia Francesa que chegaram ao Brasil para assistir o Campeonato Mundial de Futebol sediado em mais de 12 Estados do país (6). Segundo o Ministério da Saúde, entre 2014 e 2016 cerca de 1,2 milhões de pessoas foram infectadas pelo ZIKV. Embora o número de infecções seja muito expressivo, acredita-se que alguns diagnósticos possam ter sidos confundidos com pacientes infectados pelo vírus da Dengue ou Chinkungunya, pois compartilham de alguns sintomas clínicos semelhantes. Com o surto de infecção por ZIKV no Brasil iniciou-se também um surto, nunca antes relatado, de crianças recém-nascidas diagnosticadas com microcefalia e adultos diagnosticados com a Síndrome de Guillain-Barré, onde só em 2015 mais de 1200 crianças foram diagnosticadas com microcefalia, cerca de 8 vezes mais casos quando comparado com o mesmo período do ano de 2014 (7).

Umas das definições mais aceitas de microcefalia foi proposta por Böök *et al.*, que considerou que uma criança seria diagnosticada com microcéfalo se a circunferência da cabeça dela fosse menor que três vezes o desvio padrão da média da circunferência de cabeças de crianças da mesma idade e sexo (8). Durante a gravidez, se a mulher é ou está infectada pelo ZIKV, este é capaz de ultrapassar a barreira placentária, infectando e causando efeitos citopáticos às células neurais do feto. Desta maneira, as células do sistema nervoso central do feto podem ter sua morfologia, funcionalidade, metabolismo e anatomia modificada, alterando a capacidade de crescimento e desenvolvimento natural dessas células (9). Recém-nascidos diagnosticados com esta condição apresentam vários sintomas que alteram e atrasam o seu

desenvolvimento social, intelectual e motor, tais como: atraso no desenvolvimento da fala, incoordenação motora, desequilíbrio exagerado, distorções faciais e rigidez muscular.

Por outro lado, a síndrome de Guillain-Barré é definida como uma doença autoimune que afeta o desenvolvimento neural, seja do sistema nervoso central ou do sistema nervoso periférico. A relação entre o aumento de casos de infecção por ZIKV e casos de Guillain-Barré foi reportada por muitos artigos encontrados na literatura e acredita-se que após a infecção por ZIKV o vírus ataque as células neurais, modificando sua natureza, fazendo com o que o sistema imune do paciente não reconheça essas células como próprias (5). Nestes casos, o sistema nervoso tanto central como periférico é lesado, podendo desencadear sintomas como: paralisia temporária dos membros, paralisia temporária dos músculos, visão turva e dificuldade em falar. Essa capacidade e preferência de infectar células neurais, tanto nos casos de microcefalia quanto nos casos da síndrome de Guillain-Barré é definida como neurotropismo e a capacidade de causar efeitos citopáticos nessas mesmas células é definida como neurovirulência. Estudos recentes (*in vitro*) demonstraram que o ZIKV pode infectar e destruir células progenitoras neurais, neurônios e células da glia. Dentre as células mais afetas pelo vírus estão as Células Progenitoras Neurais (NPCs – Neural Progenitor Cells). Uma vez que o vírus atinge essas células os efeitos decorrentes dessa infecção podem resultar em uma menor migração celular, alteração da neurogenesis (processo de formação de novas células neurais), e consequentemente levar a morte celular (10). Além disso, testes feitos *in vivo* em camundongos confirmaram que o ZIKV ultrapassa a placenta e tem uma preferência de infecção por células neurais, demonstrando que esse tipo de infecção viral ataca e destrói células do sistema nervoso central mesmo em fetos(11). Devido aos argumentos apresentados acima, tanto pela capacidade neurotrópica do Zika vírus e seu potencial neurovirulento representados nos aumentos dos casos de microcefalia e síndrome de Guillain-Barré, é razoável crer que o ZIKV poderia regular o crescimento de células cancerígenas do sistema nervoso central, tais como as células de glioblastoma.

Glioblastoma

Glioblastoma (GBM) é o tipo de tumor mais letal do sistema nervoso central (SNC) que representam cerca de 1,4% dos canceres diagnosticados mundialmente e são responsáveis por cerca de 2,6% das mortes (12). Tumores cerebrais totalizam aproximadamente 90% dos tumores primários encontrados no SNC, destes, 50% são glioblastomas. Embora a intervenção clínica, como quimioterapia e cirurgia, possa aumentar a expectativa de vida do paciente, os glioblastomas são os tumores cerebrais mais agressivos, podendo levar à morte do paciente após apenas 3 meses de sua detecção (13). O GBM resulta principalmente da desregulação dos sinais celulares que controlam o crescimento celular, reparo de DNA e síntese proteica. Essa desregulação celular acontece, pois os pacientes manifestam alterações no gene TP53. Esse gene é responsável por produzir a proteína p53, capaz de regular funções de controle de ciclo celular, apoptose e metabolismo (14). Além disso, mais de 40% dos pacientes apresentam mutações genéticas no gene TP53, o que dificulta ainda mais o tratamento assertivo dessa doença. Sendo assim, as técnicas mais utilizadas atualmente para o combate ao glioblastoma não se mostram assertivas e específicas para o aumento da expectativa e qualidade de vida dos que sofrem com essa condição.

Devido ao fato de que as células cancerígenas são muitas vezes reconhecidas como não-próprias pelo sistema imune, uma vez que esse tipo de células é resultado de mutações genéticas, o combate ao glioblastoma se mostra ainda mais desafiador, pois deve-se considerar a fragilidade e instabilidade do sistema imune dos pacientes após o inicio das repostas humoral e celular (15). Desta maneira, uma possível infecção e ataque a essas células tumorais por uma linhagem selvagem de ZIKV poderia gerar maiores complicações aos pacientes diagnosticados com glioblastoma, agravando-se seu quadro clínico. Portanto, nesse trabalho, sugere-se a utilização de uma linhagem de ZIKV atenuado, no qual os efeitos negativos ao sistema imune e os efeitos colaterais de uma infecção viral são expressivamente reduzidos quando comparados a uma linhagem de ZIKV selvagem. Entretanto, a atenuação do Zika vírus pode diminuir seu potencial de neurotropismo, diminuindo sua capacidade de mobilidade e predileção por células neurais.

Problema o qual pode ser resolvido pela utilização das outer membrane vesicles (OMV`s).

Outer Membrane Vesicles (OMV`s)

As membranas de vesículas externas, mais conhecidas como outer membrane vesicles são membranas arredondadas, produzidas e externalizadas por bactérias Gram-negativas (16). Essas membranas são formadas durante o crescimento e colonização e são extensões naturais de suas bactérias produtoras. As OMV`s são compostas por uma bicamada lipídica que também contém lipopolissacarídeos e algumas proteínas. O envelope formado por essa membrana pode conter moléculas como peptideoglicanos, proteínas solúveis, enzimas e também uma grande gama de pequenas moléculas, tais como RNA e DNA (17). Quando as bactérias aptas a produzir essas membranas sofrem algum tipo de estresse ambiental elas externalizam as OMV`s com o intuito de transportar e proteger seu material genético e fatores de virulência. Esse sistema é uma evolução natural que viabilizou a sobrevivência de algumas bactérias, criando um microambiente que possibilitasse seu crescimento e desenvolvimento em condições desfavoráveis. Sendo assim, essas vesículas possuem função de transporte e proteção do material internalizado. Além de transportar, as OMV`s são capazes de ativar repostas do sistema imune inato e adquirido através da apresentação dos Padrões Moleculares Associados a Patógenos (pathogen-associated molecular patterns [PAMP`s]) (18). Os PAMPS`s se ligam aos Receptores de Reconhecimento de Padrões das Células Apresentadoras de Antígenos desencadeando toda a ativação do sistema imune. Um exemplo muito conhecido de microrganismo que apresenta o processo de síntese de OMV`s são as bactérias da espécie *Peseudomonas aeruginosa*. Estas são capazes de externalizarem OMV`s contendo fatores de virulência que são protegidos e transportados para as membranas citoplasmáticas das células hospedeiras (19). Quando essas vesículas chegam ao seu local de destino elas interagem e

se fundem com a membrana citoplasmática dessas células, liberando a carga transportada para dentro do citoplasma, ativando o sistema imune. O local de destino de cada OMV é definido pelo tropismo da sua bactéria produtora(20). Como exemplo, temos a bactéria *Neisseria meningitidis* que são bactérias Gram-negativas causadoras da meningite meningocócica, grave inflamação das membranas que envolvem o cérebro. Essas bactérias apresentam tropismo por células neurais, logo as OMV`s produzidas possuem como destino as células neurais do hospedeiro, fato este no qual nos levou a cogitar o uso de OMV`s de *Neisseria meningitidis* no estudo proposto (21). Desta maneira, acredita-se que a utilização de OMV`s produzidas por *Neisseria meningitidis* possa suprir o problema da diminuição do neurotropismo causados pela atenuação do Zika vírus.

Objetivos

Objetivo Geral

Determinar se o ZIKV atenuado é capaz de controlar o crescimento de células de glioblastoma.

Objetivos Específicos

Inserir o ZIKV atenuado em OMV`s de *Neisseria meningitidis.*

Observar a possível morte de células de glioblastoma por microscopia ótica.

Identificar possíveis biomarcadores de alterações metabólicas que estejam relacionadas com a morte celular das células de glioblastoma.

Avaliar o potencial antiproliferativo do ZIKV atenuado sobre diferentes linhagens celulares em cultura.

CAPÍTULO I

Metabolic alterations induced by attenuated Zika virus in glioblastoma cells

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Abstract

Background: Zika virus (ZIKV) has recently become a major matter of concern since its association with microcephaly cases in newborns was determined. From then on, ZIKV has been untiringly studied, and several scientific data have shown the virus' tropism to neural cells. Previous studies have proposed that ZIKV induced glioblastoma cells death, suggesting that ZIKV and ZIKV-associated molecules might induce intracellular biochemical alterations, and thereby be alternatives for neural cancer management. In this sense, the present contribution presents a prospective approach for glioblastomas management: producing a ZIKV-based therapy that stimulates glioblastoma control. We developed an attenuated ZIKV prototype based on bacterial outer membrane vesicles (OMV). The attenuated ZIKV was applied on glioblastoma cells, where cytopathic and cytostatic effects were evaluated. Glioblastoma cells, with and without treatment, were submitted to mass spectrometry analysis.

Results: Microscopic analysis showed cytopathic effects induced by ZIKV prototype on glioblastoma cells after 24 and 48 hours post-treatment. DNA fragmentation assay and TNF-alpha expression were indicative that ZVp induced cell damage and death. The metabolomics investigation elected 5 different biomarkers that might be associated with cell cytopathic effects, highlighting intracellular biochemical modifications induced by the attenuated ZIKV. The remarkable evidence of cell death in glioblastoma stimulated further studies that rendered a preliminary screening with other tumor cell lineages, though anti-proliferative activity test. Among the 11 tumors evaluated, prostate

and ovarian tumors were the most affected by ZIKV prototype, and other 6 cell lines also presented cytostatic effects.

Conclusions: Results ultimately demonstrated that this prototype not only emerges as a potential alternative for glioblastoma management, but may also be an important tool against other important tumors.

Keywords: Zika virus; Outer membrane vesicle; Prototype; Mass spectrometry; Cancer research.

Background

In the last two years, the absence of profound knowledge about ZIKV has encouraged intense research all over the world. Scientific investigations are stimulated mainly due to the serious impact over neural development of fetuses from mothers who were infected by ZIKV during pregnancy [1, 2], as well as the Guillain-Barre Syndrome that may affect adult infected individuals, although to a lesser extent. Recent studies have focused on disclosing intracellular processes alterations as well as the biochemical pathways modified by viral infection. Some of them have shown that ZIKV infection increased cell-cycle deregulation and cell death rates *in vitro*, in human neural progenitor cells (hNPCs), in addition to apoptosis activation associated with deregulation of DNA transcription [3]. Transcriptome analyses, evaluated by McGrath *et al*. [4] in human fetal brain-derived neural stem cells infected with ZIKV, have revealed the down-regulation of genes associated with cell-cycle and neurogenesis, while genes involved with apoptotic cell death and innate immunity were up-regulated. Most recently, mechanistic insights have been shown by Devhare et

al., who evaluated two ZIKV strains that displayed different growth rates in hNSCs. Although these two ZIKV strains presented different cellular mechanisms, both induced death, either through DNA damage, caspase-3 cleavage or increased p53 activity [5].

According to the examples cited above, the focus of ZIKV scientific research has been upon its effects on neural progenitor/stem cells, mainly due to the evidences of viral neural tropism. However, new insights have emerged: ultimately, Zhu *et al.* [6] and Lima *et al.* [7] have considered ZIKV cell death associated with tropism for progenitor neural cells as a useful approach for neural tumor management. Their work showed that ZIKV is capable of infecting and effectively killing glioblastoma stem cells in comparison to normal neuronal cells, differently from other neurotropic viruses such as West Nile virus, which induces cell death in both normal and tumor neural cells. Their findings suggest that genetically modified ZIKV would be a viable therapeutic strategy for glioblastomas control, as it is the most aggressive and chemoresistant neural tumor reported to date [6, 7].

While genetic engineering of ZIKV would be an interesting alternative as oncolytic strategy, since the exact mechanism of ZIKV neuropathogenesis is still not well-described, the exact modifications needed in viral structure to produce an assertive and safe oncolytic virus remains impaired. Although tumor management through virotherapy is a promising strategy, safety issues such as unexpected toxicities, virus mutability and transmissibility, and healthy cells elimination [8, 9] must be understood and considered, as well as the triggering factors for Guillain-Barre Syndrome, in the case of ZIKV.

Taking into account previous literature background that provide basis for the use of ZIKV specifically in neural cancer therapy, and potential safety issues concerning an oncolytic bioengineered ZIKV, we hereby propose an attenuated Zika virus prototype (ZVp) with viral fragments encapsulated into the OMVs of *Neisseria meningitidis* as an alternative to cancer management.

Results

Cytopathic effects of ZVp in GBM cells

Intending to evaluate cytopathic effects of the ZVp on neural cancer cells, human malignant U-251 glioblastoma cells (GBM) were divided into four different groups, which received distinct treatments, including a medium-only specimen (control group – CT), empty OMV, ZIKV and ZVp. All groups were evaluated through bright field microscopy in two timepoints of infection: 24h and 48h. In addition, after 24 hours of infection, all groups were also submitted to Wright staining microscopy analysis (Figure S1). Thus, the first timepoint evaluated showed that treatment with ZVp induced mild cytopathic effects, represented by morphological alterations as round, swollen cells, syncytium formation, cytoplasm fragmentation and chromatin condensation (Fig. 1d and Fig. S1d). All of these effects were milder in OMV and ZIKV groups, but non-existing in the control group (Fig. 1a-c and Fig. S1a-c). After 48h of treatment, ZVp group (Fig. 1h) presented shape-altered cells, loss of cellular integrity and notably fewer cells compared to other groups (Fig. 1e-g), which highlights substantial cytopathic effects induced in the second timepoint of treatment with ZVp.

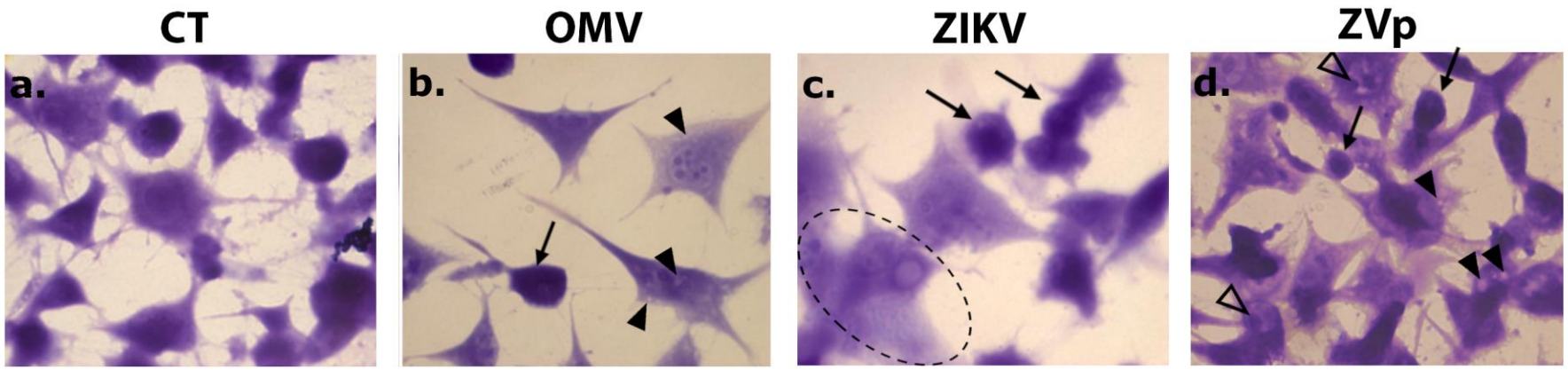

Figure S1. Optical microscopic analysis of U-251 cells under Wright staining

Representative microscopic fields of glioblastoma cell cultures submitted to Wright staining after 24 hours of treatments. Control group (CT); Empty Outer Membrane Vesicles (OMV); wild-type Zika virus (ZIKV); attenuated Zika virus prototype (ZVp). Black arrowhead: cytoplasm vacuolation; Arrow: round shape-altered cells; White arrowhead: Chromatin condensation; Dotted ellipse: multinucleated large and irregular cells. Original magnifications: x100

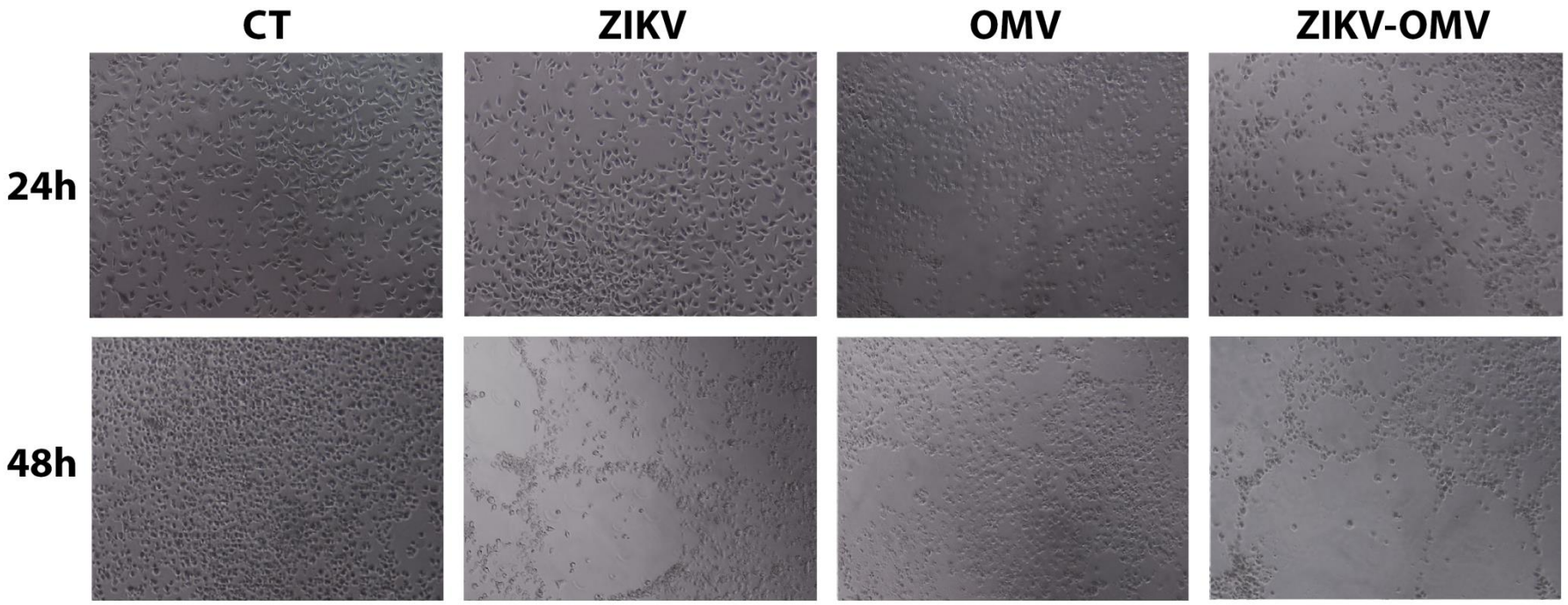

Figure 1. Cytopathic effects of the ZVp in glioblastoma cells.

Glioblastoma cells, submitted to four different treatments, were evaluated through bright field optical microscopy performed at 24 and 48 hours post-infection. (a-d) are microscopic images made at 24 hours post infection, and (e-h) are their counterparts, 48 hours post infection. The groups were: Control (CT); empty Outer Membrane Vesicle (OMV); wild-type Zika virus (ZIKV); attenuated Zika virus prototype (ZVp), respectively Scale bars, 100 µm.

In addition to cytopathic effects, it was possible to evaluate DNA fragmentation in all four groups, 24 hours after infection (Fig. S2), evidencing that ZVp-treated cells presented putatively increased DNA damage compared to all other groups. The same evaluation was not performed for the later timepoint of infection due to reduced mRNA extracted from treated samples, given that ZIKV and ZVp presented intense reduction of cells.

Corroborating with these observations, we have also evaluated TNF-alpha expression through qRT-PCR. This semi-quantitative analysis showed that ZVp presents higher TNF-alpha expression (Figure S3). Although it was not statistically significant compared to the other groups, ZVP presents an important tendency in inducing overexpression of TNF-alpha cytokine.

Figure S2.

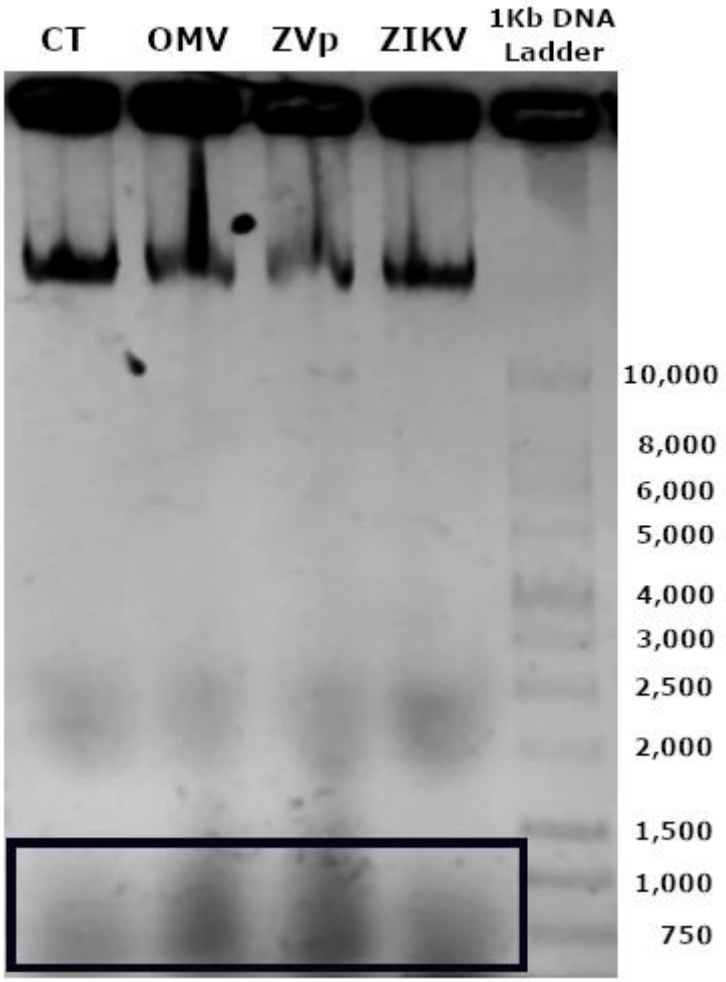

Figure S2. DNA fragmentation assay

DNA fragmentation assay in agarose gel of U-251 glioblastoma cells under different treatments: Control group (CT); empty outer membrane vesicles (OMV); Zika virus attenuated prototype (ZVp); wild-type Zika virus (ZIKV). The DNA fragments are pointed by the rectangular selection.

Figure S3.

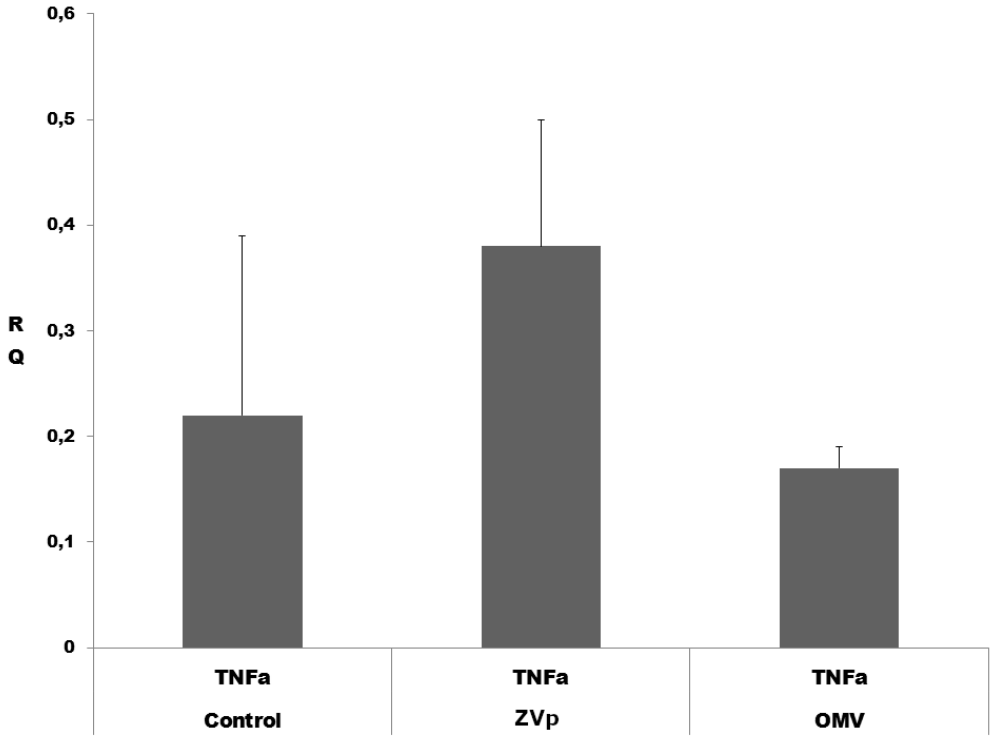

Figure S3. Expression of TNF-alpha in U-251 glioblastoma cells

The qRT-PCR technique was performed to evaluate semi-quantification of cytokine TNF-alpha mRNA in U-251 glioblastoma cells, under different treatments: Control group (CT); Zika virus attenuated prototype (ZVp); empty Outer Membrane Vesicles (OMV). RQ: Relative quantification (log of fold change in expression). Three different biological replicates were used to perform statistical evaluation of TNF-alpha expression, using GAPDH as endogenous control.

Biomarkers identification through ESI-HRMS analysis

Intending to elucidate biochemical mechanisms involved with the cytopathic effects, we performed a comparative Metabolomics analysis between glioblastoma CT and ZVp groups, after 24 hours of treatment. Intending to achieve metabolites responsible for triggering cell cytopathic effects, metabolomics analysis has prioritized to perform the biomarkers elucidation in the first timepoint of the ZVp treatment. In this way, it is possible to know exactly how to interfere in tumors to induce cell death in a further phase. The data obtained from mass spectrometric analysis were submitted to a PLS-DA, which pointed differences between metabolite composition in CT cells versus attenuated ZIKV treated cells (Figure S4). From a threshold value of 3.1 in VIP scores, biochemical markers for the ZVp-treated group were highlighted. The proposed prototype supported the election of five different biomarkers for the ZVp-treated group, illustrated in spectral Figure 2. Among elected markers we found three chlorinated phospholipids: Lysophosphatidic acid (*m/z* = 473), an oxidized Phosphatidylserine (*m/z* = 652) and a simple Phosphatidylserine (*m/z* = 586), a chlorinated metabolite of the folate pathway, 5-Methyltetrahydropteroyltri-L-glutamate (*m/z* = 675), and a deprotonated Phosphatidylinositol-3-phosphate (*m/z* = 1049; all in Table S2).

Figure S4.

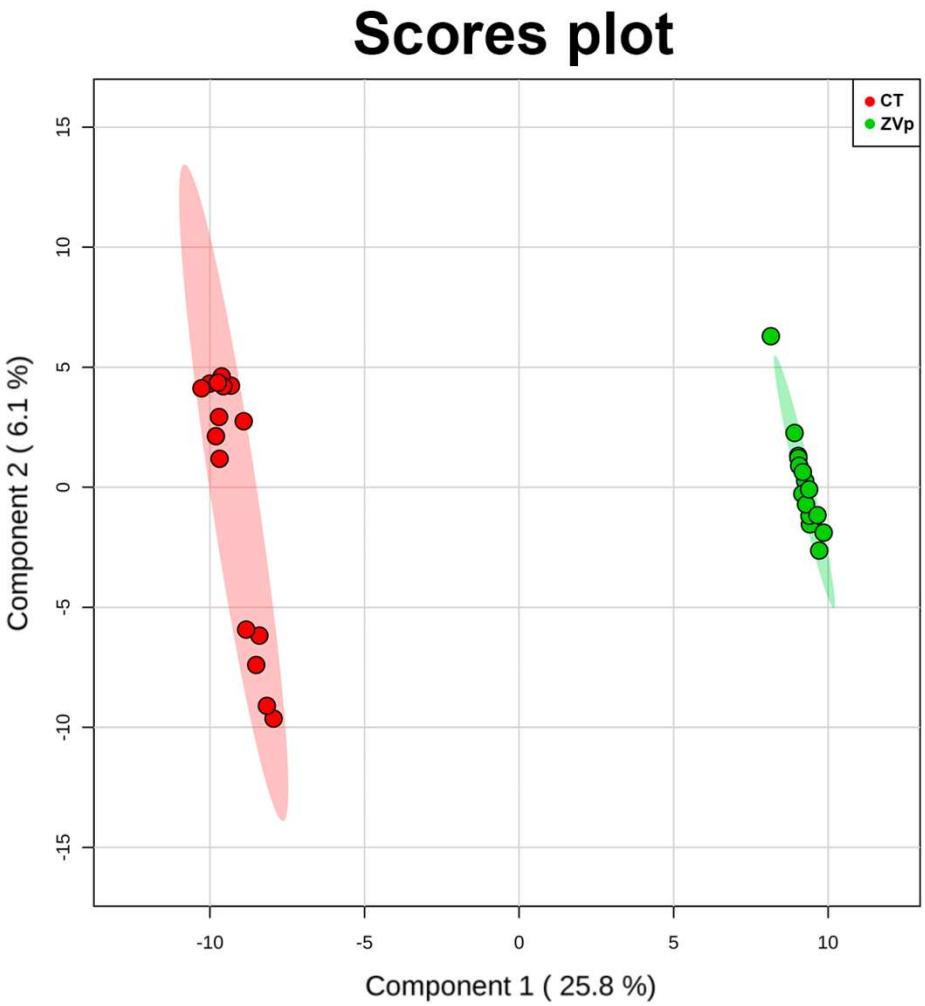

Figure S4. Statistical evidences of metabolomics differences between Control and ZVp treated groups.

Scores plot derived from PLS-DA statistical analysis between Control (CT) and attenuated Zika virus prototype (ZVp) groups; (a) 24 hours post-treatment. Each spot corresponds to one replicate analyzed, where the red spots correspond to CT group and the green ones, to the ZVp group.

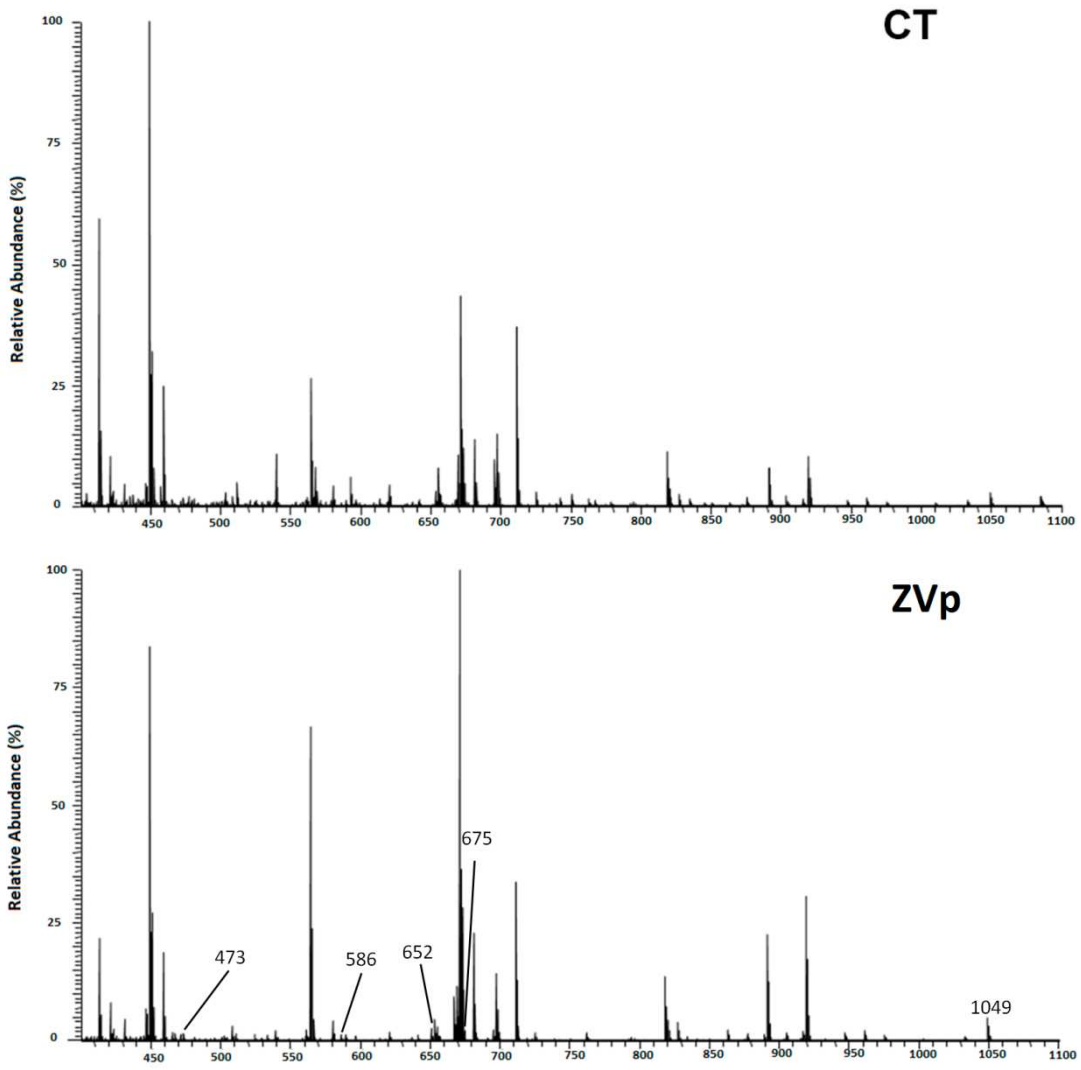

Figure 2. Representative fingerprinting of the Control (CT) and ZIKV prototype treated (ZVp) glioblastoma cells (ZIKV), 24hours post-treatment.

The imaging represents a sum of all ions within mass range *m/z* 400−1100 on negative ion mode. The ion indications correspond to the identified biomarkers shown in Table S2.

Table S2. Lipid chemical marker elected by PLS-DA VIP scores for U-251 glioblastoma cells after 24 hours of attenuated ZIKV-prototype (ZVp) treatment (negative ion mode)

Group	Ion (*m/z*)	Molecule[a]	ID[b]	Theoretical mass (*m/z*)	Experimental Mass (*m/z*)	Mass error (ppm)
ZVp	473.2	[LPA (0:0/18:0) + Cl]$^-$	MID59310	473.2440	473.2432	1.69
	586.2	[PS(20:1(11Z)/0:0) + Cl]$^-$	MID78847	586.2917	586.2908	1.54
	652.2	[PS (22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) + 3O + Cl]$^-$	MID78840	652.2295	652.2307	-1.84
	675.2	[5-Methyltetrahydropteroyltri-L-glutamate + Cl]$^-$	MID3684	675.2142	675.2147	0.74
	1049.4	[PIP3(16:0/16:0) - H]$^-$ [PtdIns-(3,4,5)-P3 (1,2-dipalmitoyl) - H]$^-$	MID61525 MID63023	1049.4175	1049.4164	1.05

Identification is based on HR-FTMS exact mass compared to the theoretical mass of each metabolite available in the online database METLIN.

Abbreviations:

[a] LPA, lysophosphatidic acid; PS, phosphatidylserine; PIP3, Phosphatidylinositol Triphosphate; PtdIns, Phosphatidylinositol.

[b] MID = METLIN ID.

Anti-proliferative ZVp assay

To investigate any possible effects of the ZVp upon proliferation in glioblastomas and expand this investigation to different tumor lines, the anti-proliferative activity of ZVp was evaluated (Figure S5), which was expressed as the sample concentration, in OMV.mL^{-1}, required to produce 50% of cell growth inhibition (GI_{50} value). Other parameter calculated was the selectivity index (SI) that is an indicator of anti-proliferative effect on normal tissues although, as an on-target toxicology indicator (the expected drug effect), it was not able to predict other systemic toxic effects (Table S1). According these parameters, the ZVp showed a selective cytostatic effect against prostate (PC-3, GI_{50} = 14.7 x 10^6 OMV mL^{-1}, SI = 40.1) and ovarian (OVCAR-03, GI_{50} = 149.5 x 10^6 OMV mL^{-1}, SI = 3.9) tumor cell lines, whilst showed potential to inhibit cell growth in 50% of tumor cell lines U251, NCI ADR/RES, 786-O and HT-29 and immortalized cell line HaCat.

Figure S5.

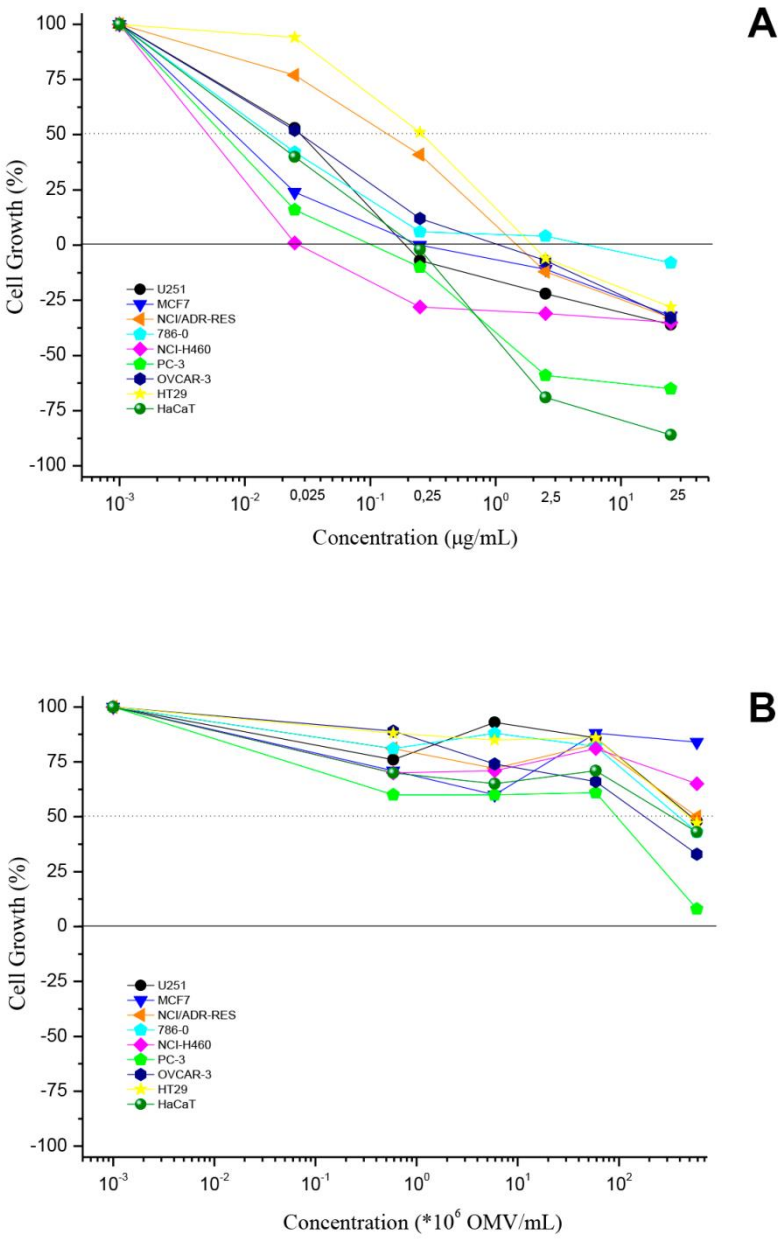

Figure S5. Anti-proliferative profile of ZVp against a panel of human tumor and non-tumor cell lines.

Anti-proliferative effect of Doxorubicin (A) and ZVp (B) over 9 cell lines after 48 hours of treatment. Doxorubicin was used as positive control for the anti-proliferative test. The effect is expressed as sample concentration (OMV.mL^{-1}) required to inhibit 50% of cell growth (GI_{50} value). Human tumor cell lines: U251 = glioblastoma; MCF-7 = breast; NCI-ADR/RES = multidrug resistant ovarian; 786-O = kidney; NCI-H460 = lung, non-small cells; PC-3 = prostate; OVCAR-03 = ovarian; HT-29 = colon; Human non-tumor cell line: HaCat = immortalized keratinocytes.

Table S1. Antiproliferative effect of attenuated ZIKV prototype (ZVp) against a panel of human tumoral and non-tumoral cell lines expressed as GI_{50} and selectivity index.

Sample	Parameter	Cell lines								
		U251	**MCF-7**	**NCI-ADR/RES**	**786-O**	**NCI-H460**	**PC-3**	**OVCAR-03**	**HT-29**	**HaCat**
Doxorubicine	**GI_{50} (ng/mL)**	25	< 25	120	< 25	< 25	< 25	27	250	< 25
	SI	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
ZVp	**GI_{50} (*10^6 OMV/mL)**	590	> 590	590	497,7	> 590	14,7	149,5	590	590
	SI	1,0	< 1,0	1,0	1,2	< 1,0	40,1	3,9	1,0	-

GI_{50}: concentration that inhibits 50% cell growth or cytostatic effect after 48h-exposition. SI: selectivity index calculated as $GI_{50\ (HaCat)}$/ $GI_{50\ (cancer\ cell\ line)}$. n.a.: not applicable ($GI_{50\ (HaCat)}$ < 0.025). Human tumor cell lines: U251 = glioma; MCF-7 = breast; NCI-ADR/RES = multidrug resistant ovarian; 786-O = kidney; NCI-H460 = lung, non-small cells; PC-3 = prostate; OVCAR-03 = ovarian; HT-29 = colon; Human non-tumor cell line: HaCat = immortalized keratinocytes.

Discussion

Results support the potential of using an attenuated ZIKV for the control of glioblastomas and other tumor cell lines, whilst bring into focus novel biochemical markers associated with tumor anti-proliferative effect induced by ZIKV particles associated with *Neisseria meningitidis* outer-membrane vesicles. The first evidence pointed out was the cytopathic effect observed after treatment of U-251 glioblastoma cells with the ZVp, even 24h or 48h post-attenuated ZIKV inoculation (Fig. 1 and Fig. S1). In addition to microscopic evidences, DNA fragmentation assay indicates that ZVp induced DNA damage in glioblastoma cells (Fig. S2), which is coherent with the tendency of TNF-alpha overexpression (Fig. S3), associated with double-stranded DNA fragmentation [10, 11]. TNF-alpha is also an important cytokine that acts in tissue homeostasis and immune response regulation [12]. Considering that this cytokine is described as tumor necrosis inductor [13], also through Caspase-3 activation [14], it is possible to infer that ZVp might be activating the TNF-alpha synthetic pathway. Therefore, it is plausible that glioblastoma cells death be induced by biochemical intracellular pathways mediated/activated through TNF-alpha.

In line with the toxic effects of ZVp observed in the present research, previous studies have shown cell survival rate and cycle alterations due to ZIKV infection in glioblastoma cells, where tumor cells infected with ZIKV showed cytopathic effects [7] and apoptosis [6]. In addition to caspase-3 activation, shown by Zhu et al., some studies have also shown that apoptosis induced by ZIKV might be associated with perturbation of Toll-like receptors-3 (TLR3)

network [15] and consequent inhibition of Sonic Hedgehog and RAS-ERK signaling [16]. Inhibition of these biochemical pathways could result in cell cycle arrest and blockage of cell proliferation, as well as induction of cell death [17, 18], which is coherent with cytostatic and cytopathic results observed in the present study.

Other results support that the ZVp may also activate other Toll-like receptors (TLRs), since bacterial outer membrane vesicles present Lipopolysaccharide (LPS), known as a potent activator of Toll-like receptor 4 (TLR4) [19]. Upon LPS-TLR4 activation, several studies have demonstrated that LPS-TLR4 interaction induces reactive oxygen species (ROS) production through activation of NADPH oxidase (Nox) [20, 21]. In addition to oxidative environment is indicative of biochemical imbalance, it may also be neurotoxic and induce cell death [22, 23]. These alterations support the oxidative ambient induced by the ZVp observed in our research, where metabolomics analysis allowed the identification of an oxidized phosphatidylserine (PS) (*m/z* = 652.2); in contrast, a non-oxidized PS (*m/z* = 586.2) was also identified. It is well-known that PS exposure on the outer layer of cell membrane is indicative of apoptotic cells, working as specific markers for phagocyte recognition and removal [24, 25]. Therefore, PS identification may also be a consequence of cell death induced by ZVp.

In addition to ROS, inducible nitric oxide synthase (iNOS) is upregulated in microglia cells when under pathological conditions as viral infections, corroborating an intracellular environment of oxidative stress [22, 26, 27]. Nitric oxide (NO), a product of iNOS, has been reported as a regulator of carbon flow

through the folates pathway, as NO binds to Cobalamin (oxidation) and inhibits its role as a cofactor of methionine synthase (MS) [28, 29]. 5-Methyltetrahydropteroyltri-L-glutamate, an elected biomarker for the ZVp-treated group, is involved in homocysteine remethylation to methionine mediated by MS. Considering that MS activity is inhibited by oxidized Cobalamin under oxidative conditions, the metabolite 5-Methyltetrahydropteroyltri-L-glutamate may accumulate, thereby indicating alterations in the one-carbon transfer reactions mediated by the folate pathway. The folate-mediated synthesis of methionine is crucial for DNA methylation and production of purines and pyrimidines, as folate is responsible for providing one-carbon (methyl) blocks for nucleotide synthesis [30, 31]. Hence, it is possible to infer that our ZVp was able to negatively interfere on DNA methylation and nucleotide synthesis, favoring cell cycle arrest, chromosome instability and, ultimately, cell death [32-34].

Moreover, phosphatidylinositol trisphosphate (PIP3) was also identified in glioblastoma cells treated with the ZVp. A recent contribution by our group has also observed phosphatidylinositol phosphates among ZIKV biomarkers [35], corroborating our findings. Usually, under pathogens challenge, Akt-mTOR might be activated through TLR4 stimulation, what happens under LPS stimulation [36-38]. However, according to Q Liang, Z Luo, J Zeng, W Chen, SS Foo, SA Lee, J Ge, S Wang, SA Goldman, BV Zlokovic, et al. [39], ZIKV presents two non-structural proteins (NS4A and NS4B) that suppress the Akt-mTOR pathway signaling. Thus, it is possible to infer that TLR4 activates phosphatidylinositol-3-kinase (PI3K), which increases PIP3 availability, but without success in signaling cascade proceeding, taking into account that ZIKV

proteins are capable of inhibiting Akt-mTOR complex, the final target of PIP3. Therefore, phosphatidylinositol trisphosphate accumulates due to the constant TLR4 activation by attenuated ZIKV prototype antigens and becomes an evident biomarker in our experiment.

Taking into account that microglia cells were the target of mass spectrometric analysis in this study, we must consider their particular characteristics. Microglia cells are the neuroprotective and immunocompetent ones among glial cells [40, 41], and can be activated by extracellular stimuli and under injury conditions, represented here by the ZVp. Once activated, microglia cells undergo for profound morphological and biochemical changes, culminating with synthesis of bioactive molecules. Lysophosphatidic acid (LPA) is a bioactive phospholipid and is abundantly present in brain cells [42, 43]. Under microglia activation, LPA concentration increases due to oxidative damage, and phospholipases activation, which catalyzes LPA production [44, 45]. Concerning the present study, LPA was one of the biomarkers identified for glioblastoma cells under treatment with the ZVp. In face of the oxidative previous explanation and the infectious environment simulated by the attenuated ZIKV, it is plausible to infer that LPA production was increased. Some studies have also shown that LPA induces cell death by modulating redox environment or through upregulation of Tumor Necrosis Factor receptor [46, 47]. Therefore, it is possible that the primary redox imbalance induces LPA synthesis and subsequently LPA itself contributes to intensify the intracellular oxidative environment. Furthermore, TNF has also been related as a neuronal cell death inductor stimulated by ZIKV [48]. ZVp presents ZIKV inactivated by heat, thereby not excluding the presence of ZIKV antigens, even inactivated; hence, it

is possible that ZIKV antigens may still sensitize cellular response and induce immune response. Thus, LPA may be involved in induction of cell cytopathic effects observed in our study.

In addition to metabolic alterations induced by the ZVp over glioblastoma cells, we have also intended to confirm its cytopathic effects over the same cell line and evaluate its potential over several other sorts of tumors. Therefore, we have performed a preliminary anti-proliferative activity test over glioblastomas and eight other different cell lines. This first test confirmed the anti-proliferative effect of the ZVp over GBMs, since it showed 50% of cytostatic effect. Interestingly, the attenuated ZIKV prototype cytostatic effect has also been observed in six other tumors and one immortalized cell lines. Noteworthy, the most affected cell lines were prostate and ovarian tumor cells, where cytostatic effect was most evident. It has been recently observed that ZIKV presents tropism for urogenital cells [49-51], and our results show that the ZVp presented a similar tropism, even after inactivation by heat. Although prostate and ovarian cells were the most affected, the attenuated ZIKV prototype still presents potential to interfere with other 6 cell lines that have also shown cytostatic effect, mainly glioblastoma, which is the most studied cell line with respect to ZIKV infection.

Conclusions

All these findings allowed us to propose a number of altered biochemical pathways in tumor cells during the ZVp treatment. The most interesting data shown by the present study is the possibility of using an attenuated ZIKV based into ZIKV for management of glioblastomas, expanding its potential to be

applied for management of different tumors. The advantages of using this method consists of the immune protection provided by it and the lower cost compared to virus genetic modification. This study is a window that opens for further researches, such as *in vitro* adjustment of attenuated ZIKV doses, *in vivo* tests of viability into animals' tumors and applications in human cancer management.

Methods

Attenuated ZIKV prototype development

N. meningitidis (C2135); *Aedes albopictus* (C6/36) and glial cells (M059J) were the cell lines used in this study, all obtained from (INCQS – FIOCRUZ – National Institute for Quality Control - Oswaldo Cruz Foundation, Rio de Janeiro, RJ and Cell Bank). The ZIKV strain used in the present study corresponds to Brazilian ZIKV strain (BeH823339, GenBank KU729217), which was isolated from a patient in the State of Ceará (Brazil) in 2015. The viral strain was gently provided by Professor Doctor Edison Durigon (Biomedical Sciences Institute, University of São Paulo).

Each cell line was grown under the following conditions: M059J cells line were submitted to cultivation with RPMI1640 medium supplemented with 10 % of fetal bovine serum (FBS) and 1% of antibiotics (tetracycline 1µg/ml, levofloxacin 1µg/ml, erythromycin 3µg/mL in hydroalcoholic 50% solution); C6/36 cell line was cultured and infected according to CFOR Melo, DN de Oliveira, E de Oliveira Lima, TM Guerreiro, CZ Esteves, RM Beck, MA Padilla, GP Milanez, CW Arns and JL Proença-Modena [52]; *N. meningitidis* was grown at 37ºC under 5% of CO2 in agar GCB (Difco). C6/36 or M059J were used to

replicate ZIKV when cell confluence achieved 70%. Aliquots of 1mL ZIKV were prepared when viral cytopathic effect (CPE) reached until 75 %, and were stored at -86ºC.

OMVs Extraction and OMV- ZIKV conjugated prototype

Preparation of OMVs from *Neisseria meningitidis* was performed according to Alves et al. and stored at -80ºC before use [53]. Aiming to obtain the ZVp, ZIKV grown in C6/36 were used to infect M059J cells; soon after ZIKV was fused with OMV vesicles from *N. meningitidis*. For this, different concentrations of OMV were added to M059J ZIKV-infected cells during different times points, in order to obtain the best attenuated ZIKV preparation. The resulting supernatants containing OMV fused with ZIKV were collected and inactivated at 56°C for 1 hour. Nano Tracking Analysis (Zetasizer Nano) was used to analyze the prototype (ZVp) preparations.

Cell culture and attenuated ZIKV prototype treatment

Human malignant U-251 glioblastoma cells (SIGMA product no. 09063001), provided by Professor Marcelo Lancellotti, were seeded in T-25 culture flasks, cultured in RPMI 1640 medium and incubated at 37°C with 5% of CO_2. Upon 80% of confluence, each test flask (attenuated ZIKV group) was submitted to treatment with $1x10^{10}$ ZVp.mL^{-1}, while the control group (CT) underwent the same conditions, except for prototype treatment. The experiment resulted in three biological replicates for each condition studied. Optical microscopic examinations were performed at 24 and 48 hours post-treatment and a Zeiss Observer A1 microscope was used to capture bright field images, which were processed by Zeiss' AxioVision 4 software. After each time point of treatment, the cells were detached from each flask surface through

trypsinization and submitted to sample preparation according to Melo *et al.* [35]. At last, the analytical technique of choice was mass spectrometry, which was performed with electrospray ionization and direct injection into a high-resolution mass spectrometer (ESI-HRMS).

Mass spectrometry analysis

After sample preparation, the analytical technique of choice was mass spectrometry, which was performed through electrospray ionization and direct injection into a high-resolution mass spectrometer (ESI-HRMS). Spectra were acquired using an ESI-LTQ-XL Orbitrap Discovery (Thermo Scientific, Bremen, Germany) with a nominal resolution of 30,000 (FWHM). The sample's ions were analyzed at the mass range of 400 to 1100 *m/z*, in the negative ion mode, comprising a total of five analytical replicates per group. Spectra were analyzed using XCalibur software (v. 2.4, Thermo Scientific, San Jose, CA).

Cellular death assays and Cytokine Real-Time PCR

The experiments involving the glioma death were performed as follows: DNA fragmentation and morphological cells analysis were made by agarose gels staining with ethidium bromide and Wright staining viewed in light microscopy, respectively. The TNFα production was performed by qRTPCR using as endogenous control the GAPDH gene.

Statistical analysis

The method of choice to investigate differences between groups was Partial least squares discriminant analysis (PLS-DA), a statistical method that uses multivariate regression techniques to extract variables that may evidence these differences. The selection of ions which were characteristic for each group was carried out based on the impact that each feature presents in the model, i.e. the analysis of VIP (Variable Importance in Projection) scores. The important chemical markers for each group were selected based in cutoff threshold established as a VIP score greater than 3.1. PLS-DA and VIP scores analysis were performed using the online software MetaboAnalyst 3.0 [54, 55].

TNF-alpha mRNA semi-quantification was performed through ANOVA and experiments were performed in triplicate for all evaluated groups, which were: CT, OMV and ZVP. The data shown in the graphs represent the means ± standard errors.

***In vitro* antiproliferative assay**

The anti-proliferative activity of ZVp was investigated over glioblastoma cells (U251). Aiming to verify the potential of using attenuated ZIKV for control of tumors in general, seven other human cancer cell lines were also evaluated (MCF-7 = breast; NCI-ADR/RES = multidrug resistant ovarian; 786-O = kidney; NCI-H460 = lung, non-small cells; PC-3 = prostate; OVCAR-03 = ovarian; HT-29 = colon). Tumor cell lines were kindly provided by Frederick Cancer Research & Development Center, National Cancer Institute, Frederick, MA, USA. Cell proliferation was also evaluated using a non-tumor cell line HaCat

(immortalized human keratinocytes), kindly provided by Dr. Ricardo Della Coletta (University of Campinas- UNICAMP, Brazil).

Stock cultures were grown according to WA Roman Junior, DB Gomes, B Zanchet, AP Schönell, KA Diel, TP Banzato, AL Ruiz, JE Carvalho, A Neppel and A Barison [56] and the attenuated ZIKV (1.18×10^9 OMV mL^{-1}) was prepared directly diluted in the complete medium, affording the final concentrations of 0.59, 5.9, 59 and 590 x 10^6 OMV mL^{-1}. Doxorubicin (final concentrations of 0.025, 0.25, 2.5 and 25 µg·mL-1 in complete medium) was used as positive control.

Cells in 96-well plates (100 µL $well^{-1}$, inoculation density: 3.5 to 6 x 10^4 cell·mL^{-1}) were exposed to different concentrations of sample and control (100 µL.$well^{-1}$) in triplicate, for 48 h at 37°C and 5% of CO_2. The anti-proliferative test and the colorimetric assay were performed according to Monks and Skehan, respectively, and GI_{50} values were determined using Shoemaker software [57-59]. The selectivity index (SI) was calculated according to Muller and Milton [60].

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ML was responsible for ZVp development, maintenance of cellular culture and ZVp administration on glioblastoma cells. MZD and EOL performed sample collection, mass spectrometry experiments and wrote the manuscript. MZD, EOL and DAD were responsible for microscopic analysis and photographs. ALTGR and GG conducted the anti-proliferative tests. MZD, EOL and DNO wrote the manuscript. DNO, CFORM, TMG and KNM performed data analysis and manuscript review. RRC idealized all experiments and managed the research group. MZD, EOL and DNO contributed equally during the research development and conclusion. All authors read and approved the final manuscript.

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Discussão geral

As análises das alterações metabólicas induzidas por Zika vírus atenuado em células de glioblastoma é de extrema importância para uma melhor compreensão de possíveis alternativas para o combate desse tipo de tumor. Assim, o desenvolvimento de novas metodologias de elucidação do mecanismo de combate do ZIKV ao glioblastoma é de extrema relevância. As metodologias e resultados gerados nesse trabalho indicam o potencial uso do Zika vírus atenuado como possível método de controle anti-proliferativo do glioblastoma e também outros tumores.

A associação das partículas virais com as vesículas de membranas externas (OMV`s) de *Neisseria meningitidis* se mostrou eficiente para resolver o problema de perda de neurotropismo que o processo de atenuação gera às células que passam por esse processo. A perda de mobilidade do ZIKV impossibilitaria que a infecção ocorresse de maneira eficaz e comprometeria o processo de entrada do vírus dentro da célula e assim o controle do crescimento do tumor. Entretanto, a atenuação viral é de extrema importância, pois os pacientes que são diagnosticados com glioblastoma sofrem devido à fragilidade de seu sistema imune. Sendo assim, se a infecção fosse feita com uma linhagem de Zika vírus selvagem, o paciente, já debilitado, teria todos os efeitos colaterais de uma infecção viral e possivelmente seu quadro clínico se agravaria. Dessa maneira, a atenuação e associação com as OMV`s tornam-se necessárias.

Ao analisar os resultados das figuras e tabelas pôde-se observar os efeitos citopáticos e citostáticos que o ZVp causou nas células de glioblastoma quando crescidos em co-cultura tanto 24 ou 48 horas de inoculação (Fig. 1 e Fig S1). As fotos da microscopia demonstram que as partículas virais induziram alterações morfológicas e anatômicas nas células de glioblastoma. É possível também compreender que o ZVp, além de ser capaz de produzir biomarcadores que indicam a sua capacidade de induzir efeitos apoptóticos nas células de glioblastoma (Tabela S2), também provocam a fragmentação do DNA destas células (Figura S2), demonstrando o potencial deste método no controle do crescimento tumoral, não só de glioblastoma, mas também de

outros tipos (Figura S5). O ensaio anti-proliferativo do ZVp resultou em um efeito citostático de mais de 50% em sete tipos de tumores e em células imortalizadas. As células mais afetadas por esse efeito foram os tumores de próstata e ovário, demonstrando que além do glioblastoma o Zika vírus atenuado pode controlar o crescimento de outros tipos tumorais, ressaltando a importância e relevância do trabalho.

Conclusão

O trabalho alcançou seu objetivo, pois as metodologias e resultados apresentados demonstraram-se muito eficiente para o controle de crescimento de células de glioblastoma. Além disso, este estudo possibilita a expansão dessas metodologias para outros tipos de tumores.

As vantagens da utilização da uma linhagem de Zika vírus atenuado com a inserção em OMV`s de *Neisseria meningitidis* consistem na proteção imune e eficácia que o método apresenta, além do custo reduzido quando comparado com as técnicas que são atualmente utilizadas.

O estudo também abre uma janela de oportunidade para futuras pesquisas *in vivo* para ajustes e testes de viabilidade de doses e aplicações.

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Anexos

Autorização da revista

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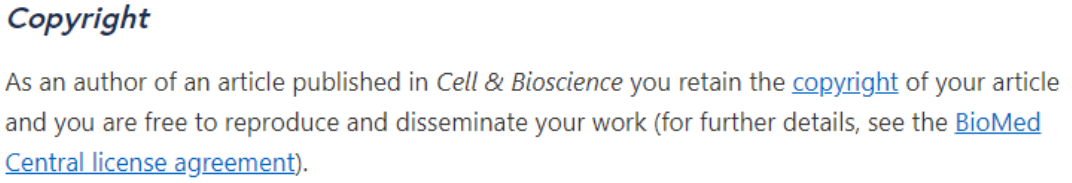

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