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Padronização de Protocolo de Imunofluorescência em Microscopia Confocal para Avaliação de Alterações Bioquímicas e Citomorfológicas induzidas pela Neurotoxina 6-Hidroxidopamina à Linhagem Celular SH-SY5H

Monografia de Conclusão de Curso para obtenção parcial do grau de Bacharela em Biomedicina com Habilitação em Bioquímica.

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> *"Estou entre aqueles que pensam que a ciência tem grande beleza. Um cientista em seu laboratório, não é apenas um técnico: é também uma criança colocada diante de fenômenos naturais que podem impressioná-lo como a um conto de fadas."*
>
> Marie Curie – Cientista, primeira mulher a receber um Prêmio Nobel.

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RESUMO

Um dos grandes desafios no estudo sobre a Doença de Parkinson (DP) é a elucidação da fisiopatologia dos neurônios dopaminérgicos da via nigro-estriatal durante a progressão da DP. Utilizando a linhagem celular de neuroblastoma humano SH-SY5Y, diferenciada em linhagem tipo neurônio dopaminérgico, como modelo *in vitro* para estudos moleculares da DP são descritos estudos que avaliam, bioquímica e morfologicamente, os eventos descritos na patologia da DP. Sabe-se que a oxidação da proteína cofilina-1 medeia disfunção mitocondrial e apoptose em células tumorais e, pode potencialmente desempenhar um papel importante na morte de células neuronais induzidas por neurotoxinas como a 6-hidroxidopamina (6-OHDA), uma droga comumente usada para indução de DP em animais. Estudos com esta linhagem demonstram a translocação de cofilina-1 do citosol para a mitocôndria durante tratamento com 6-OHDA, entretanto nenhum estudo utiliza técnicas de microscopia para avaliação desde fenômeno. A microscopia é uma das técnicas descritas como menos invasiva, por manter a estrutura celular, permitindo a observação das características bioquímicas e morfológicas das células. Contudo, ainda são necessários aprimoramentos e a implementação de bons protocolos para utilização de equipamentos já bastante difundidos no meio científico, como a microscopia, possibilitando a utilização dessa ferramenta de estudo para diversos tipos celulares, cultivos, tecidos e amostras. Dessa forma, este trabalho se propõe a descrever um bom protocolo de imunofluorêscencia para microscopia confocal que tenha foco na morfologia celular e do seu conteúdo interno, como a distribuição de cofilina-1 nas células SH-SY5Y quando desafiada por 6-OHDA, utilizando parâmetros bioquímicos para validar o protocolo proposto. Nosso objetivo em desenvolver um protocolo de imunofluorêscencia para esta linhagem celular foi bem sucedido e validado pela apresentação de fragmentação mitocondrial durante tratamento com a neurotoxina, como descrito na literatura. Entretanto, não foi possível avaliar o comportamento de cofilina-1 pelo método utilizado neste momento, sendo necessárias repetições. De toda forma, este trabalho aborda pontos importantes a serem questionados ao trabalhar com as técnicas de

imunofluorêscencia, orientando o leitor formas e pontos críticos, tornando possível adaptar um protocolo de imunofluorêscencia a todo tipo de amostra biológica para microscopia confocal.

Palavras-Chave: Microscopia Confocal, Imunofluorêscencia, Cofilina-1, Doença de Parkinson, 6-Hidroxidopamina, Linhagem SH-SY5Y.

1 INTRODUÇÃO

1.1 Cofilina-1: Uma proteína multifuncional envolvida na sinalização pró-apoptótica

ADF/cofilina é uma família de proteínas de ligação a actina, que está envolvida na despolimerização dos filamentos de actina. São proteínas muito conservadas entre os eucariotos. A família de proteínas ADF/cofilina é expressa em todas as células, e está disposta em três formas nos mamíferos: ADF (actin depolymerizing factor), cofilina-1 (n-cofilina ou não muscular) e cofilina-2 (m-cofilina ou muscular). A cofilina-1 liga-se a actina-G (monomérica), bem como à actina-F (filamentosa) [1], sendo um conhecido fator da regulação dinâmica de despolimerização dos filamentos de actina. A cofilina liga-se lateralmente ao filamento de actina, provocando uma torção do filamento, deixando-o mais compacto. Esse estresse mecânico torna a actina friável e mais fácil de quebrar. A cofilina liga-se preferencialmente aos filamentos que contém ADP (adenosina difosfato), ou seja, é mais eficiente na despolimerização dos filamentos velhos, garantindo uma substituição rápida dos filamentos de actina na célula [2, 3]. Além da função junto ao citoesqueleto, a cofilina-1 é descrita em diversos outros processos dentro da célula como a translocação para o núcleo em resposta a certos estímulos físicos ou químicos. [4]. Outra nova função atribuída a cofilina-1 é o controle que esta proteína exerce sobre a apresentação de antígenos dependente de apresentação de MHC de classe II, citando a cofilina-1 com regulador da apresentação de antígenos [5].

E ainda a cofilina-1 é descrita como parte da sinalização de apoptose, foco deste trabalho. Estudos de proteômica mostram que a cofilina-1 desfosforilada é translocada para a mitocôndria através da oxidação se seus resíduos de cisteína, na presença de diferentes oxidantes, sendo proposta como parte da sinalização pró-apoptótica induzindo *swelling* mitocondrial e libertação do citocromo c pela mediação da abertura dos poros de transição de permeabilidade mitocondrial independente de Bax [6, 7]. Este papel da

cofilina está sendo estudado, mas ainda não há um consenso quanto ao exato papel desta proteína na sinalização de apoptose [8], contudo já é descrito o envolvimento de cofilina-1 mediando apoptose em diversos processos celulares como a morte por exitotoxicidade em neurônios glutamatérgicos [9], resposta a compostos antitumorais [10] e outros.

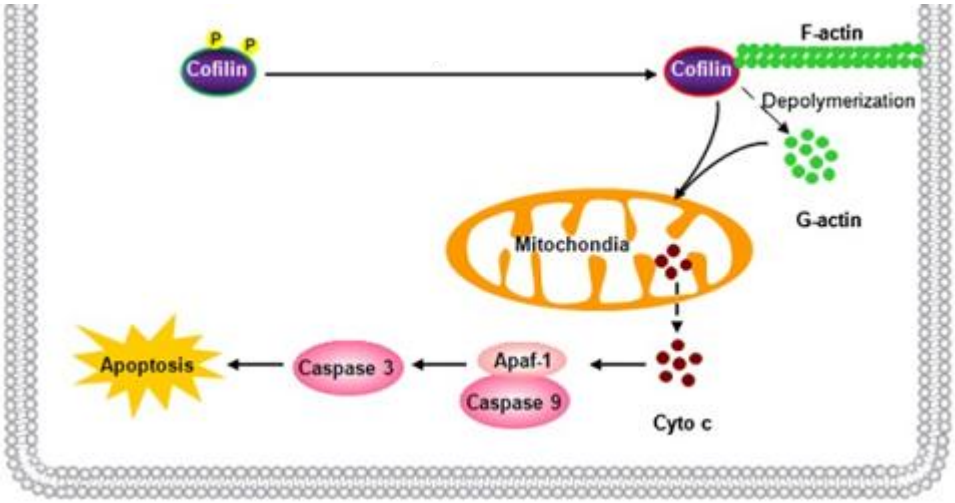

Figura 1: Representação esquemática do mecanismo de inicialização de apoptose via dano mitocondrial mediado por cofilina-1. (Adaptado de Li G.B., *et al*, 2013).

1.2 Características da Apoptose

A apoptose é um processo fisiológico, mas pode ser também patológico [11]. A apoptose pode ser desencadeada por fatores externos como internos. Fatores como o Fator de Necrose Tumoral (TNF), Fator de Crescimento β de Transformação (TGF-β), alguns neurotransmissores, radicais livres, oxidantes, radiações UV e ionizantes podem desencadear a apoptose. Alguns destes atuam sobre os receptores de membrana da célula, deflagram a apoptose ao recrutar e ativar a cascada das caspases. Internamente, a apoptose também pode ser executada por oncogenes, supressores tumorais como a proteína p53, antimetabolitos de privação de nutrientes [12], e cada vez mais são descritos outras componentes celulares como iniciadores intracelulares de apoptose, a exemplo da cofilina-1, como já citado.

Antigamente, a apoptose era descrita como uma reação irreversível, contudo, já há trabalhos mostrando que mesmo que a célula entre em processo apoptótico, há estágios aos quais a reversão é possível [13]. Quando uma célula recebe um sinal para iniciar o processo de apoptose, a primeira consequência é um aumento da permeabilidade da membrana mitocondrial externa, permitindo a liberação do citocromo c que normalmente fica confinado no espaço intermembrana. O citocromo c liberado, ativa a caspase 9, responsável pela degradação de proteínas durante a apoptose. Quando a apoptose é desencadeada a célula apresenta algumas características morfológicas e bioquímicas específicas como:

- Perda do potencial de membrana mitocondrial ou *swelling* mitocondrial – Há alterações da permeabilidade dos canais transmembrana, e a integridade da membrana é rompida. Além da liberação do citocromo c, íons como Ca^{2+} também são liberados bem como muito dos prótons, dessa forma, a mitocôndria perde seu potencial eletroquímico [14].

- Fragmentação do DNA – Pela ativação das endonucleases dependentes de Ca^{2+} e Mg^{2+}, o DNA é fragmentado em pedaços e a cromatina agrega-se. O núcleo continua delimitado pela carioteca, mas não está mais íntegro (padrão apresentado é diferente da cariorrexe, apresentada em situações de necrose, quando o DNA é também fragmentado, mas há perda dos limites nucleares).

- *Blebbing* na membrana – Resulta de alterações na membrana celular, resultantes da translocação de determinadas moléculas como fosfatidilserina da membrana celular, da face citoplasmática para a face externa. Essas alterações promovem bolhas na membrana, sem o rompimento. Essa alteração sinaliza a fagocitose por células do sistema imune.

- Formação de corpos apoptóticos ou fragmentação citoplasmática – A etapa final da apoptose resulta na clivagem celular. Estas vesículas ligadas à membrana originam as chamadas bolhas citoplasmáticas, contendo organelas (quando os fragmentos contem material nuclear, são chamados corpos apoptóticos) para que os restos celulares sejam rapidamente fagocitados.

1.3 Linhagem celular SH-SY5Y como modelo de estudos *in vitro* de Doença de Parkinson

1.3.1 Doença de Parkinson

A Doença de Parkinson (DP) é a segunda doença neurodegenerativa mais comum na população (sendo a Doença de Alzheimer a primeira). Esta doença foi descrita pela primeira vez por James Parkinson em 1817 e caracteriza-se por ser uma doença neurodegenerativa progressiva crônica, de sintomatologia predominantemente motora como bradicinesia, tremor de repouso, rigidez e distúrbios na postura. A DP também está associada com uma diversidade de sintomas não motores, como hiposmia (diminuição do olfato), movimentos oculares rápidos, distúrbio de comportamento do sono, alterações de personalidade, dor, parestesias (sensações cutâneas na ausência de estímulo) e depressão podem estar presentes e se manifestarem antes dos sintomas motores. Em fase tardia, há o agravo dos sintomas com instabilidade postural e quedas, o congelamento da marcha, fala e dificuldades de deglutição. A DP tem um início gradual entre as idades de 50 e 70 anos, de progresso lento, culmina na morte do indivíduo de 10 a 20 anos mais tarde [15].

A fisiopatologia da DP envolve a perda progressiva de neurônios dopaminérgicos da *substantia nigra pars compacta* que conduzem a desenervação da via nigroestriatal o que acarreta significativa redução de dopamina ao nível do corpo estriado (núcleo caudado e putâmen). Os sintomas só se manifestam quando há perda de 50-60% das células dopaminérgicas e quando há 80-85% menos liberação de dopamina no corpo estriado [16, 17]. E diferentemente de outras doenças neurodegenerativas, na DP a degeneração se mantêm restrita aos neurônios da *substantia nigra pars compacta*. Embora a causa da deterioração progressiva destes neurônios dopaminérgicos seja desconhecida, investigações genéticas estão fornecendo pistas sobre a etiologia e patogenia desta doença. Mas mesmo antes dos estudos genéticos da DP, análises bioquímicas de cérebros *post-mortem* estabeleceram a disfunção mitocondrial, o estresse oxidativo e agregação de proteínas como os principais fatores da patogênese desta doença [18, 19].

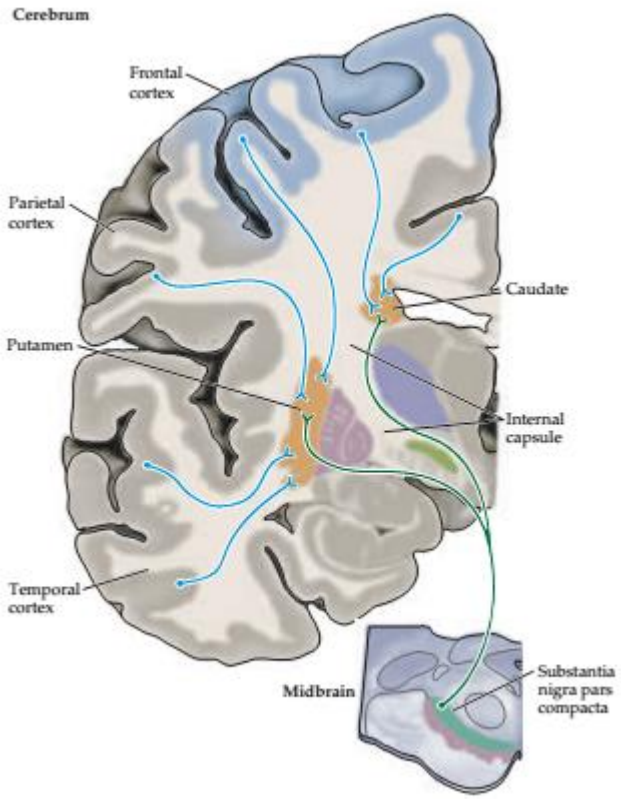

Figura 2: Representação dos Núcleos da Base afetados pela degeneração dos neurônios dopaminérgicos na Doença de Parkinson em linhas verdes (Retirado de Purves, D. *et al*. Neuroscience, Cap 17; 3ed. Sunderland: Sinauer, 2008).

A importância da mitocôndria na patogênese da DP aumentou devido à identificação de mutações nos genes que codificam proteínas mitocondriais [20]. Outra causa discutida para a patogênese da DP é o estresse oxidativo. Existem evidências mostrando o dano oxidativo ao DNA, às proteínas e aos lipídios na *substantia nigra pars compacta*, além da diminuição dos níveis de antioxidantes, como a glutationa (GSH) [21-24]. A disfunção mitocondrial afeta um grande número de vias celulares, culminando em dano nos componentes citoplasmáticos e em morte celular. Algumas funções e morfologia anormais foram observadas na mitocôndria em algumas formas da DP. E além desses fatores, os neurônios dopaminérgicos da *substancia nigra pars compacta* apresentam canais de cálcio voltagem dependente, assim a organela sequestra o cálcio intracelular, resultando na liberação de citocromo c e de fatores pró-apoptóticos, como o fator de iniciação da

apoptose (IAF), que inicia uma cascata de eventos culminando na morte do neurônio dopaminérgico [20].

Como ainda não existem marcadores biológicos ou um padrão definitivo de imagens, o diagnóstico da DP é atualmente feito através de critérios clínicos rigorosos. Contudo, estudos clínicos e histopatológicos com base no material do banco de cérebros do Canadá e do Reino Unido demonstraram que os médicos diagnosticam a doença incorretamente em cerca de 25% dos pacientes. Isso denota a necessidade de ampliar as pesquisas que visem aprimorar a compreensão da doença e de seu tratamento visto que a incidência desta doença, assim como outras doenças neurodegenerativas, está se elevando com o aumento da expectativa de vida [25].

1.3.2 Modelo experimental: *Linhagem de Neuroblastoma Humano SH-SY5Y*

Pela impossibilidade de obtenção de biopsias humanas de pacientes com DP, a maior parte dos estudos sobre esta doença são executados em modelos animais, como ratos e camundongos. Mesmo havendo grande sintenia (organização genética muito semelhante) entre estes modelos animais e humanos, a não realização dos estudos em células humanas se torna um empecilho para a aplicação de novas descobertas na clínica médica.

A linhagem de neuroblastoma humano SH-SY5Y é um subclone derivado do neuroblastoma SK-N-SH. Essas células, quando em cultura, se assemelham a neuroblastos do sistema simpático, apresentando morfologia epitelial com citoplasma escasso [26]. Células dessa linhagem estão em estágios iniciais da diferenciação neuronal, o que permite com que estas células sejam diferenciadas *in vitro* em células tipo neurônio. Muitos estudos mostram que as células de neuroblastoma SH-SY5Y podem ser diferenciadas em neurônios dopaminérgicos através do tratamento com ácido retinóico [27]. Esse composto é uma a neurotrofina responsável pela estimulação de receptores nucleares, que ativam genes responsáveis pela diferenciação e pela maturação neural. Outro elemento de diferenciação celular *in vitro* é a diminuição da concentração de soro fetal bovino (SFB),

que induz a parada da proliferação celular [28]. A linhagem SH-SY5Y é proposta como modelo para a Doença de Parkinson por se assemelhar a uma célula dopaminérgica, exibindo várias das peculiaridades fisiológicas, bioquímicas e morfológicas de tais neurônios, como o formato estrelado com projeção de neuritos alongados e as características bioquímicas, que servem como marcadores neuronais, como a enolase neurônio específica, proteína nuclear de neurônio, sinaptobrevina e sinaptofisina, tirosina hidroxilase, (enzima específica de neurônios dopaminérgicos envolvida na rota de produção de dopamina a partir da L-DOPA) e DAT (transportador de dopamina) responsável pela recaptação do neurotransmissor na fenda sináptica. Por estas características, o protocolo de diferenciação por sete dias com ácido retinóico e soro fetal bovino a 1% em meio para o cultivo da linhagem SH-SY5Y foi escolhido como modelo *in vitro* para estudos da Doença de Parkinson neste estudo [29].

1.3.3 Neurotoxina 6-Hidroxidopamina

Atualmente, a maior parte das pesquisas que induzem DP utiliza a neurotoxina 6-Hidroxidopamina (6-OHDA) [30, 31]. Esta neurotoxina foi a primeira usada em modelos animais da DP [32]. Desde então, a 6-OHDA é utilizada tanto para estudos *in vivo* como *in vitro* desta doença. Ainda, os níveis desta neurotoxina estão elevados no soro de pacientes com DP, por isso, diferentemente das outras toxinas, ela pode ser encontrada fisiologicamente [33, 34]. 6-OHDA é metabólito da dopamina e a sua estrutura é muito semelhante a esse neurotransmissor. Dessa forma, a toxina possui uma alta afinidade pelo transportador de dopamina (DAT). Assim, a 6-OHDA é capaz de entrar no neurônio dopaminérgico. Uma vez dentro do neurônio, a 6-OHDA se acumula no citosol e ocorre a sua auto-oxidação, promovendo a formação de espécies reativas de oxigênio, bem como a diminuição dos níveis de antioxidantes [31]. Esta droga também pode acumular-se na mitocôndria, onde inibe o complexo I da cadeia respiratória [34]. Os neurônios dopaminérgicos da *substantia nigra pars compacta*, além de conter níveis significantes de dopamina, também apresentam H_2O_2 e ferro. Uma reação não enzimática destes três elementos

pode levar à formação de 6-OHDA [35]. Estudos anteriores mostraram que a morte celular causada pela 6-OHDA é devida à apoptose. Ainda, acreditava-se no envolvimento do poro de transição de permeabilidade mitocondrial neste tipo de morte celular [36].

1.3.4 Utilização de 6-OHDA no modelo *in vitro* SH-SY5Y

Por mais de 30 anos são publicados trabalhos que utilizam a linhagem SH-SY5Y, (com diferentes protocolos de diferenciação ou até mesmo em estado proliferativo) e a neurotoxina 6-OHDA, descrevendo mecanismos da patogênese da DP ou outros estudos de biologia celular [37]. Há na literatura uma grande variação dos valores de concentração de 6-OHDA utilizados para causar dano às células SH-SY5Y. Dessa forma assumimos o valor de 15 µM como DL_{50} (concentração necessária para causar dano letal a 50% das células em cultivo) descrito por Lopes *et al*, 2010. Nesse mesmo trabalho é descrita a perda do potencial de membrana mitocondrial na linhagem SH-SY5Y devido ao tratamento com 6-OHDA pelo método do ensaio de JC-1.

Ao que se refere aos estudos que associam esta linhagem a função de cofilina-1, dos poucos trabalhos publicados, as metodologias utilizadas variam entre os ensaios clássicos de bioquímica como o ensaio de MTT e western blot até a transfecção destas células com diferentes plasmídeos. Contudo, nenhum trabalho descreve ou utiliza algum método que vise à avaliação do comportamento de cofilina-1 com relação a apoptose nas células SH-SY5Y quando desafiadas por 6-OHDA. Assim, propomos a microscopia como método de estudo para a elucidação do papel da cofilina-1 durante apoptose na linhagem de neuroblastoma humano SH-SY5Y.

1.4 Utilização da microscopia como ferramenta de estudo em biologia celular

> "O que podemos aprender sobre as células depende das ferramentas à nossa disposição e dos avanços recentes em biologia celular, surgidos devido à introdução de novas técnicas. Portanto, para compreender a biologia celular contemporânea é necessário entender parte de seus métodos".

Texto retirado de Biologia Molecular da Célula – Bruce Alberts

Compreender os fenômenos celulares e bioquímicos perpassa compreender as técnicas à nossa disposição e aprimorar sua utilização através da busca de melhorias em protocolos e reagentes.

A microscopia tem seu início por volta do começo do século XVII quando Kepler propôs a construção de um conjunto de lentes, o microscópio composto [2]. Contudo, também é dado o crédito a Zacharias Janssen pela criação do primeiro microscópio em 1590 [38]. Em 1665, Robert Hooke descreveu pequenos poros na cortiça, que os chamou de células, nome que mudou de significado, mas perdura até hoje [39]. Somente no início do século XIX, Schleiden e Schuwann visualizaram que tecidos vegetais e animais são agrupados celulares em 1838 [40]. E no fim do século XIX foram desenvolvidos os primeiros corantes histológicos.

Atualmente temos disponíveis diversos tipos de microscópios com diferentes fundamentos físicos e resoluções bastante potentes. O correto alinhamento das estruturas do microscópio é fundamental para determinação de uma ótima resolução, com descrito por Köehler [41]. Entretanto, os microscópios óticos possuem uma limitação própria de resolução. Nenhuma estrutura menor que o comprimento de onda da luz pode ser distinguida, tornando assim, impróprio visualizar estruturas que não possuam entre si uma distância mínima de 0,4 µm. A cor emitida, ou seja, visualizada pelo homem, influi nesta capacidade resolutiva. Ao visualizar estruturas coradas em vermelho, a distância mínima distinguível é de 0,7 µm. O limite de separação pelo qual dois objetos ainda podem ser vistos como distintos é chamado limite de resolução [42].

O preparo da amostra também é uma questão que exige muita atenção. Resíduos de gordura, fibras de celulose e a presença de cristais são os artefatos mais comuns em microscopia e podem ser evitados com uma boa limpeza da lâmina e da lamínula. O tampão, a forma, o número e a intensidade das lavagens entre cada etapa do protocolo, podem resultar na perda da amostra (descolamento das células da superfície), formação de cristais e depósitos de agregados de anticorpo ou corantes, gerando pontos de intensa marcação, o que prejudica a imagem que obteremos.

Outra etapa crucial do preparo da amostra é a fixação. A fixação permite que as células se tornem permeáveis a corantes e anticorpos e produz uma ligação cruzada entre as macromoléculas, de maneira que sejam estabilizadas e presas à sua posição como uma foto, retratando o estado celular naquela situação. Alguns dos mais antigos procedimentos de fixação envolvem ácidos ou solventes orgânicos, como o álcool. Os procedimentos atuais envolvem aldeídos reativos, em particular formaldeído e glutaraldeído, que formam ligações covalentes com grupos de aminoácidos livres de proteínas.

Muitos dos corantes utilizados são compostos químicos que coram as células e tecidos através de reações químicas ou afinidades físico-químicas, como é o exemplo dos tradicionais corantes hematoxilina e eosina, que são sais em que seu componente cromógeno possui características básicas ou ácidas que se ligam a estruturas de caráter físico-químico oposto [43]. Dessa forma, estes corantes não são específicos. Para tornar mais específica a visualização de determinadas estruturas, foram desenvolvidas diversas técnicas como as que aliam anticorpos a fluoróforos para marcar com extrema especificidade determinados componentes celulares. Esta técnica, chamada de imunofluorêscencia, é utilizada desde 1942, e possibilita distinguir estruturas internas da célula através da marcação com anticorpos específicos para determinada estrutura (anticorpo primário) e anticorpos anti-anticorpo específico (anticorpo secundário), este conjugado a uma molécula fluorescente. [44, 45]

Moléculas fluorescentes são compostos que são excitáveis, absorvendo energia de um comprimento de onda específico e emitem luz de um determinado comprimento de onda maior (ou seja, de menor energia). Algumas vezes, a molécula fluorescente não é específica e há ligação desta na superfície de vidro ou plástico, ou em outras estruturas que não são alvo do estudo. Ocorrendo o que se chama “*background*”. As ligações inespecíficas dos anticorpos ou compostos fluorescentes utilizados que se ligam ao suporte sólido são devidas às lavagens inadequadas que favorecem a formação de acúmulos fora da célula.

Para realizar este tipo de técnica em microscopia, é necessária a utilização de microscópios capazes de excitar as moléculas fluorescentes,

utilizadas como marcadores, em seu comprimento de onda específico, o microscópio com estas características chama-se microscopio de fluorescência. Este, assim como o microscópio óptico, é um conjunto de lentes de aumento que possui uma lâmpada geralmente de xenônio ou mercúrio, e um conjunto de filtros que seleciona a luz que atinge a amostra (comprimento de excitação) e outro conjunto que seleciona a luz obtida da amostra (comprimento de emissão).

Um dos problemas inerentes da microscopia de fluorescência é definir exatamente a posição espacial da estrutura marcada pelo fluoróforo na amostra. Secções de tecidos com mais de 2 µm de espessura possuem, de forma mínima, mas considerável, uma terceira dimensão. A amostra ao receber a luz é excitada e emite a fluorescência de diferentes planos focais o que, por vezes, interfere na visualização da imagem, por dar um aspecto "borrado" à estrutura marcada. Este problema pode ser corrigido de duas formas: a deconvolução e a obtenção de secções ópticas. A deconvolução é a correção matemática das imagens, prontamente processadas por um computador que calculará a contribuição da desfocagem na formação da imagem e que corrigirá digitalmente esse defeito de óptica, alcançando o limite teórico de resolução. Assim obtemos uma imagem límpida, com menos borrões. [2] A outra forma, obtendo secções ópticas da amostra, foi possibilitada pela criação de um microscópio que utiliza um *Laser* (*Light Amplification by Stimulated Emission of Radiation*) como fonte de luz, que através de um conjunto de espelhos seleciona os feixes emitidos pela amostra, permitindo que apenas um plano focal da amostra seja capturado pelo fotomultiplicador. Este equipamento é chamado Microscópio Confocal, inventado por Marvin Minsky em 1955 [46, 47]. A terminologia "secções ópticas" se refere ao fato de que nesta metodologia são obtidas imagens de secções da amostra, de forma não invasiva, através da coleta seletiva de informações de um único plano focal por vez. O microscópio ilumina apenas um ponto da amostra de aproximadamente 1 μm^3 de volume, e dessa forma, espécimes relativamente espessas podem ser visualizados nos volumes sucessivos através da aquisição de uma série de secções ao longo do eixo óptico (Eixo Z) do microscópio.

O aspecto fundamental para a abordagem confocal é a utilização de técnicas de filtragem espacial para eliminar a luz para fora de foco. O ponto de luz é focado por uma lente objetiva no plano focal desejado na amostra, a luz que passa através do espécime é focada por uma segunda lente objetiva e após, um orifício permite a passagem da luz de somente um foco, o mesmo que o emitido, ou seja, confocal um ao outro. Qualquer luz que passe o segundo orifício atinge um fotomultiplicador de baixo ruído, que gera um sinal que é relacionado com a intensidade da luz a partir da amostra. O segundo orifício impede a entrada de luz proveniente de um plano acima ou abaixo do obturador. O uso de filtragem espacial para eliminar a luz fora de foco, em amostras que são mais espessas do que o plano do foco, é a chave para a abordagem confocal.

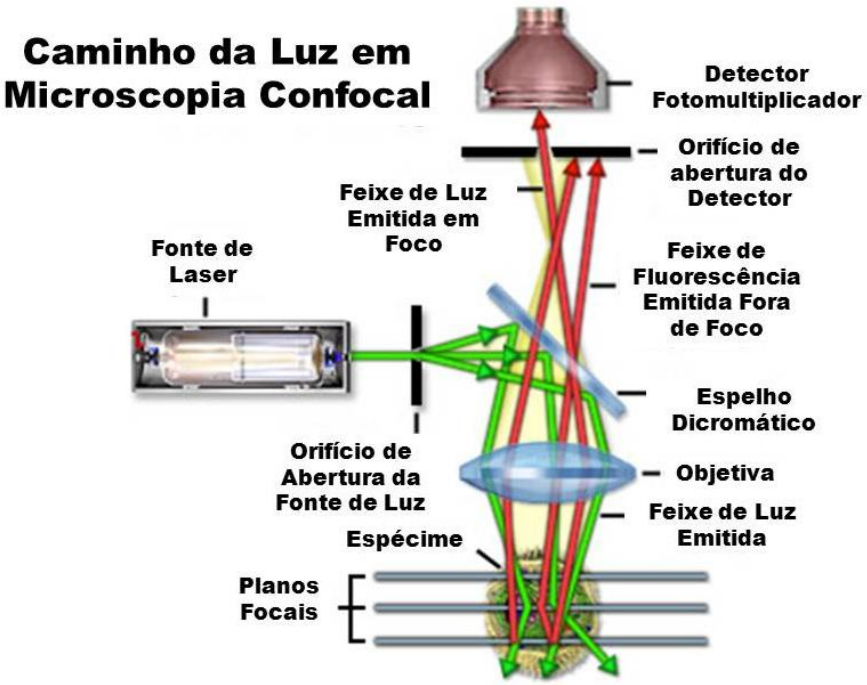

Figura 3: Figura esquemática do funcionamento do Microscópio Confocal (adaptado de Nikon website).

O microscópio confocal tem tido um aumento de popularidade nos últimos anos, em parte devido à relativa facilidade com a qual as imagens, de altíssima qualidade, podem ser obtidas a partir de amostras preparadas para a microscopia de fluorescência convencional, e ao crescente número de aplicações em biologia celular que dependem de imagem. Na verdade, a

tecnologia confocal está provando ser um dos avanços mais importantes já alcançados em microscopia óptica [48-52].

A utilização da microscopia de fluorescência não está limitada a apenas análises qualitativas da amostra. Cada vez mais, os dados de imagem são utilizados para fornecer informações semiquantitativas com o auxílio de softwares como *ImageJ* (*Java-based image processing program*) [53]. Entre as poderosas ferramentas para analisar processos no contexto fisiológico de células vivas intactas, a microscopia de fluorescência é citada como a mais promissora, pois cada vez mais estas técnicas estão sendo aplicadas a experimentos de genômica funcional. Dessa forma, a microscopia de fluorescência está em transição: de uma técnica qualitativa para quantitativa e de alto rendimento [54, 55]. Podem ser citadas como novas aplicações da microscopia de fluorescência, técnicas como: a expressão de proteínas recombinantes fluorescentes, que é a conjugação de genes que codificam proteínas autofluorescentes [56]; reconstrução de imagem 3D, que utiliza todos os dados espaciais obtidos da microscopia confocal e através um programa de computador organiza as informações para gerar uma imagem em 3D da célula [57, 58]; *time-lapse* (análise por microscopia em tempo real) que é formação de vídeos da célula em tempo real, recurso muito utilizado quando se deseja analisar efeitos celulares transcorridos com tempo com modificação morfológica, como por exemplo, a apoptose, divisão celular e outros [56]; FRET (*Fluorescence Resonance Energy Transfer*) são proteínas com moléculas fluorescentes que são complementares a outros fluorocromos também ligados a proteínas quando próximas a no máximo 10nm sendo então possível avaliar interações entre proteínas, como interação ligante-receptor [59]; FRAP (*Fluorescence Recovery After Photobleachin*g) e FLIP (*Fluorescence Loss in Photobleaching*), a fim de observar o movimento de materiais intracelulares através da fotodegradação da fluorescência em um ponto e avaliando a recuperação do sinal no local onde um *laser* incidiu, dessa forma pode se examinar a fluidez e a direção do deslocamento das moléculas e continuidade de membranas [60, 61]; e por fim a colocalização que é um método bastante abrangente e utilizado em diversas situações, e que está presente em praticamente todos os métodos citados anteriormente. A colocalização objetiva visualizar a presença de

diferentes componentes na célula simultaneamente. Este resultado é obtido através da utilização de fluoróforos de diferentes cores de emissão, marcando estruturas diferentes. Como exemplo da utilização da técnica de colocalização, Cremer *et al*. desenvolveram a teoria dos territórios cromossômicos basicamente com a utilização das técnicas de fluorescência e colocalização dos genes no núcleo [62, 63]. As imagens que serão capturadas pelo fotomultiplicador no microscópio confocal, seleciona um comprimento de onda por vez – canais de imagem - assim, apresenta apenas uma cor a cada imagem. Porém, a união de diferentes cores sobrepostas nos permite inferir que as estruturas marcadas, estão muito próximas ou interagindo entre si. Como limitação, a colocalização não permite visualizar cores muito próximas no espectro da luz, a contribuição de um comprimento de onda no outros gera uma sobreposição na imagem que não é fidedigna ao estudo que se propõe.

A partir dessas informações, notando-se que a microscopia confocal possui diversas vantagens metodológicas que possibilitam avaliações citomorfológicas e bioquímicas da célula; que a morte dos neurônios dopaminérgicos, na patologia da Doença de Parkinson se dá por apoptose [36, 64]; que a neurotoxina 6-OHDA induz morte celular por este mecanismo de morte celular programada [29, 65], e que cofilina-1 é descrita como indutor intracelular de apoptose pela oxidação de seus resíduos de cisteína na presença de oxidantes, este trabalho se propõe a observar o fenômeno, da translocação de cofilina-1 para o interior da mitocôndria nas células da linhagem SH-SY5Y diferenciada. Para tanto, técnica de microscopia confocal será utilizada, e um protocolo de imunofluorescência para este modelo *in vitro* para estudos da Doença de Parkinson será estabelecido.

2 ARTIGO CIENTÍFICO

An immunofluorescence confocal microscopy method for detection of cofilin-1 in an *in vitro* model for Parkinson's disease studies

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ABSTRACT

Background: The physiopathology of Parkinson´s disease (PD) has not been completely understood. Mitochondrial dysfunction and oxidative stress have been strongly related in pathogenesis of PD and it is known that oxidation of cofilin-1 mediates this events in tumor cells by oxidants and could play a role in neuronal cell death by 6-hydroxydopamine (6-OHDA). Using the SH-SY5Y cells line following a protocol of differentiation into a dopaminergic neuron-like cells as an *in vitro* model of DP, we studied on confocal microscopy differences in cell morphology. The confocal microscopy has been one of the most popular and promising research technique, but it is necessary improvements in the protocols depending of sample. To study the behavior of cofilin-1 was developed an immunofluorescence protocol for Confocal Microscopy.

New Method: To establish an efficient immunofluorescence protocol for SH-SY5Y were used morphological and biochemical parameters to validate the protocol developed.

Results: We developed an efficient immunofluorescence protocol for SH-SY5Y cells, allowing label cofilin-1, cytochrome c, cell nucleus, and

mitochondria for future studies on confocal microscopy. Through the protocol developed, it was possible to establish the best conditions for immunolabeling. It was possible to characterize the location of some organelles in the cell correlating with the cell state and also view the mitochondrial fragmentation phenomena in response to the neurotoxin treatment.

Comparison with Existing Method(s): This protocol has developed to attend a specific cell line, although describe critical steps for the technique guiding the researchers, making possible adapt for other biological samples, because in this work is approached the essential steps of immunofluorescence.

Conclusions: The immunofluorescence is a method that allows observes the cell state minimizing the effects arising from technique maintaining the cell characteristics. It is make possible obtain many information about the cell physiopathology. In differentiated SH-SY5Y cell line was possible to note mitochondrial fragmentation in presence of neurotoxin 6-OHDA, which support the literature and allowed future studies to value the role of cofilina-1 in DP.

Keywords: Mitochondria, Confocal Microscopy, immunofluorescence, Parkinson´s Disease, cofilina-1, SH-SY5Y

Highlights

- Confocal microscopy is an important tool, which allows several biochemical and cellular studies based on cell morphology, however for each sample it should be established the best experimental conditions, due to the peculiarities of each cell type or specimen.
- We established an immunofluorescence protocol to study the cofilin-1 protein and its behavior when the cell was stressed by neurotoxins, such as 6-OHDA.
- We found an intense mitochondrial fragmentation when differentiated SH-SY5Y cells were treated with 6-OHDA.

1. INTRODUCTION

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease. This chronic disturb causes severe motor dysfunction, such as bradykinesia, resting tremor, rigidity, postural instability, and also affects autonomic function and cognition. There are not biological markers or a standard diagnostic imaging, and therefore about 25% of the patients with PD are incorrectly diagnosed (World Health Organization, 2006). The molecular mechanisms underlying neurodegeneration of the nigrostriatal pathway during the progression of PD has not been completely understood, although several factors, including mitochondrial dysfunction, oxidative stress and apoptosis have been strongly related to contribute to cell death in PD (Jenner, 2003; Schapira AH, 2011).

The protein cofilin-1 is an example of protein that is involved in different functions in the cell, depending on the situation. It is known that oxidation of cofilin-1 mediates events like cell damage by apoptosis in tumor cells through oxidation of cofilin-1's Cys residues, which cause lost of its affinity for actin, and translocation to the mitochondria, inducing swelling and cytochrome c release by mediating opening of the permeability transition pore (Klamt et al., 2009). Our hypothesis is that cofilin-1 may play a role in neuronal cell death when it is induced by neurotoxins, such as 6-hydroxydopamine (6-OHDA) (Lopes FM *et al*., 2010).

6-OHDA is the most used toxin in experimental models of PD (Bové J and Perier C, 2012). This neurotoxin cause apoptosis of dopaminergic neurons in *substantia nigra pars compacta,* reproducing an important pathological feature of PD (Li *et al*., 2012) and the levels of this neurotoxin are increased in the serum of patients with DP (Schober, 2004). Because this neurotoxin has similar structure to dopamine, it shows high affinity for the dopamine transporter and for this reason selectively destroys dopaminergic and catecholaminergic neurons. Inside the neuron, 6-OHDA accumulates in cytoplasm and undergoes non-enzymatic auto-oxidation, promoting free radical formation (Bové J and Perier C, 2012).

Nowadays, the Confocal Microscopy method has been a tremendous explosion in the popularity due in part to the relative ease with which extremely high-quality images can be obtained from specimens prepared for conventional optical microscopy, and to great number of applications in many areas of current research interest. Indeed, the confocal technology is proving to be one of the most important advances already achieved in optical microscopy (Amos and White, 2003), because through this technique is possible to visualize in intact living cells, the effects of the biochemical reactions (Ellenberg, 2006). For this reason, this study aims to develop an immunofluorescence protocol to confocal microscopy to study the behavior of cofilin-1 within SH-SY5Y cells when challenged by the neurotoxin 6-OHDA and contribute with literature about the involvement of cofilin-1 in apoptosis, a possible therapeutic target for Parkinson's disease.

2. MATERIALS AND METHODS

2.1. Cell culture and differentiation

Exponential growing human neuroblastoma cell line SH-SY5Y, obtained from ATCC® (Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium High-glucose/F12 medium (1:1 v/v) containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic mix. Cells were maintained at 37ºC in a saturated humidity atmosphere containing 5% CO_2. After 24h of cell plating the differentiation was induced by lowering the FBS in culture medium to 1% plus retinoic acid (RA) at 10 µM during 7 days (Lopes F.M. et al., 2010).

2.2. Immunofluorescence Protocol

2.2.1. Preparation of coverslips and plate the cells

To use the confocal microscope, the cells must be plated in coverslips or another plate with thickness below than 0,16 mm. The preparation of slides and coverslips is a crucial part of the process, because many dyes have affinity for fats, which soiled the image. In this work, three kinds of cleaning were performed using weak acids, organic solvents, alcohols and detergents. In order to determine the best method to clean the coverslips, we establish

immersion the coverslips in hot water (>60°C) with a neutral detergent, such as extran® 5% (v/v) (Merck KGaA, Darmstadt, Germany), wash with distilled water until complete removal of the detergent and dipping the coverslips in alcohol 70% at least 12h. The drying should be made in drying oven upper then 30ºC. Coverslips are sterilized into ultraviolet light by 30 min and put on sterile 12 well culture plate. Tweezers must be used in all cleaning process to avoid touching the coverslips with hands. This process decreases the bubbles in glass, reduces fats in the surface of the coverslip and enables the adhesion of cell without addition of substrates such as poly-L-lysine, because this cell type adhered easily to glass and plastic surfaces. However, surfaces coated with poly-L-lysine are indicated of all cells types, because this polymer blocks the glass auto-fluorescence.

The cells are trypsinized when they reach 75% of confluence and plated on coverslips. This culture is established as a standard on 25.000/cm^2, however, as this concentration is too high to view in microscope, the cells must be plated in order to avoid any overlapping of their structures. Therefore, it defaults to a reduction of 10% at a concentration of plated (if you used treatments that cause cell death, you should consider the lethal dose). That way, the cells maintain in contact with other without overlap the cells and formation of big grouped cells. SH-SY5Y cells are suspended in medium and plated on the coverslip, just on glass area, keep on 37º C and 5% CO_2 for 1h. After, adding the medium to complete the normal volume of medium. After 24h, starts the differentiation protocol. All cells have to be adhered in the surface to start the protocol, otherwise during the washes of immunofluorescence protocol the cells will be throwing it away. In case of use the proliferative cells, you must not start the immunofluorescence protocol before the 24 hours of adhesion, because cells need to stabilize on the matrix in this period to overcome the stress caused by trypsinization.

2.2.2. Reagents

2.2.2.1. Buffer

When you choose the washing buffer, it is important to consider use the smallest amount of salt as possible, without containing pH indicators, such as

phenol red, because this compound form crystal easily and there is auto-fluorescence. The most commonly buffer that is used for confocal microscopy is the phosphate buffered saline (PBS). We work with PBS and Hank's Balanced Salt Solution with phenol red (HBSS), however PBS is better than HBSS in immunofluorescence protocol.

2.2.2.2. Fixatives

The purpose of fixation is to preserve biological samples as similar to natural state as possible. This process prevents postmortem degeneration and autolysis, inhibit hydrolytic enzymes, and harden the tissues, thus facilitating subsequent staining of tissues or cells. Fixatives often alter the cells or tissues on a molecular level to increase their mechanical strength or stability to subsequent treatment as staining. A good fixative must kill the cell quickly without shrinkage or swelling, penetrate the tissue rapidly and inhibit autolysis. Fixation is usually the first stage in immunofluorescence protocol to prepare a sample for microscopy. The choice of fixative and fixation protocol is a crucial step of the process because establishes generalized molecular bonds within the cell. For example, when we work with antibodies, which bind to a specific protein target, prolonged fixation can chemically mask these targets and prevent antibody binding.

There are three types of fixatives according to the chemical properties of the compounds, although we test two classes of fixatives: crosslinking fixative and denaturing fixative. We used paraformaldehyde, the most commonly crosslinking fixative, that interacts primarily with the residues of the basic amino acid lysine and tend to preserve the secondary structure of proteins and significantly protecting amounts from denaturation the tertiary structure as well. Glutaraldehyde, another crosslinking fixative, must not br used on confocal microscopy because this fixative is too much autofluorescent.

In this work we also used methanol:acetone (1:1 v/v). This solution is classified as denaturing fixative, because acts on reducing the solubility of protein molecules by disrupting the hydrophobic interactions which give the tertiary structure of proteins. Figure 1 shows the differentiated SH-SY5Y cells line fixed by different fixatives.

It was chosen paraformaldehyde as fixative and defined a previous fixation with paraformaldehyde 2% (m/v), solubilized in PBS (pH 7.4) and medium for 20 min, just before fixation with paraformaldehyde 4% for more 20min further, samples were washed using PBS.

2.2.2.3. Mitochondria Dye

Membrane-potential-dependent dyes such as Rhodamine 123, tetramethylrhodamine methyl ester (TMRM), and tetramethylrhodamine ethyl ester (TMRE) are useful as long as the mitochondria maintains its negative membrane potential. However de commercial MitoTracker® Orange CMTMRos is a chloromethyl-X-rosamine, lipophilic cationic orange-fluorescent dye that stains mitochondria in live cells; and its accumulation is dependent upon membrane potential, and maintained in cell after aldehyde-based fixation (Chazotte, 2011). The cell-permeant MitoTracker® Orange probes contain a mildly thiol-reactive chloromethyl moiety for labeling mitochondria which are excited in 554 nm, and emitting fluorescence at 576 nm. This probe is very well establish as a sensitive mitochondrial marker when the organelle are in oxidative stress, have being a good indicator of relative changes in mitochondrial membrane potential (Pendergrass et al., 2004).

For this reason, MitoTracker® Orange CMTMRos from Molecular Probes®, Inc. (Eugene, OR, USA) (MTO) was chosen as a mitochondria marker in this work. MTO was test in cells fixed with methanol:acetone and paraformaldehyde. The dye becomes permanently bound to the mitochondria, thus remaining after the cell is fixed with both fixatives. We stained the cells at concentration of 400 nM in buffer at 37ºC for 30 min as recommended by the manufacturer. However, we perceived inespecific binding of MTO in the nucleus (what is impossible in theory) and in the coverslip, because this dye is lipophilic and stain any fat residual that is on the coverslip in high concentrations. So, is very important to clean very well the materials that are used in the work when MTO is used. In the last performed test, we used MTO a concentration of 200 nM, which diminished the background but the problem was not resolved. More tests are necessary to standardize a MTO protocol.

There are some articles, which describe MTO as inhibitor of the mitochondrial respiratory chain at complex I by permeability transition pore opening by CMTMRos, with concomitant depolarization, swelling, and release of cytochrome *c*, after staining cells with CMTMRos, in high concentrations. (Scorrano et al., 1999). So, the use of this dye requires a strict standardization according to the study that will be conducted.

2.2.2.4. Nuclear Dye

We stained the cell's nucleus with Hoechst® 33258 (Invitrogen®, Inc., Carlsbad, CA, USA) to marker all cells, independent of labeling with antibodies or dyes. This probe is cell permeable nucleic acid stains and the fluorescence of these dyes is very sensitive to DNA conformation and chromatin state in cells. Consequently, they can detect gradations of nuclear damage and is often used to distinguish condensed pycnotic nuclei in apoptotic cells and for cell cycle studies. These bisbenzimidazole derivatives are supravital minor groove–binding DNA stains with adenine/thymine selectivity.

The MitoTracker® and Hoechst® stock solutions are stable for at least 6 months when refrigerated. For long-term storage the stock solution can be aliquoted and stored at ≤ –20°C (Molecular Probes, 2005).

We used a concentration of 1 µg/mL in buffer at 37ºC for 30 min and after the cells washed twice with buffer. Hoechst® 33258 is excited in 352 nm (ultraviolet frequency) and emitted fluorescent at 576 nm (blue).

2.2.2.5. Antibodies

Tests were conducted on primary and secondary antibody to determine the best concentrations for the technique, in order to determinate each amount antibody, each fluorophore and describe the differences between them. It is always necessary to standardize the antibodies that will be used. Frequently the concentrations recommended by the manufacturers are not necessarily the best. We tested many concentrations and determine the best concentrations being as primary and secondary antibodies, as shown in Figure 2. It was used in this work Mouse anti-cytochrome c (10 µg/mL) from Invitrogen® (Carlsbad, CA, USA) and Rabbit anti-cofilin-1 (0,5 µg/mL) from Abcam® (Cambridge, England). The concentrations used are half that

recommended by the manufacturers. As secondary antibody, we used Alexa Fluor® 635 and Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) Antibodies and Alexa Fluor® 568 and Alexa Fluor® 405 Goat Anti-Mouse IgG (H+L) Antibodies from Molecular Probes®, Inc. (Eugene, OR, USA) and determined a concentration 1:250 in buffer as the standard. Sometimes, the same antibody with the same concentration, but conjugated with different fluorophores have different intensities. But also, the same antibody and fluorophores in different concentrations show dissemblance. So, it is important to test all combination before performing the experiments. The choice of the antibody must consider the resolution limit of microscopy, because the colors which are emitted change the limit of resolution. So if you want see a small structure or subtle differences in the cell after a treatment, for example, it is important choose a fluorochrome between blue and green emission, because zone in the electromagnetic spectrum have the smaller wave-length in the visible light spectrum, therefore have the best resolution.

After the fixation and removed all the fixative, the cells was washed three times with 0,2% Triton-X 100 to permeabilize membranes, shaking for 5 min each wash. Non-specific binding were blocked with 1% BSA in PBS incubate for 1h at 4ºC with 1% BSA in PBS. Cells were incubated with the on primary antibodies overnight at 4°C in shaker. After washing with PBS and 0.02% Triton-X100, cells were incubated with secondary antibodies for 1h at 4ºC, and further washed twice with PBS. In order to remove all salts crystals and prevent the formation of crystals, it is recommended a last wash with distilled water.

2.2.2.6. Mounting Medium

The coverslips are removed from wells and placed on slide glasses with a drop of Fluoromount™ Aqueous Mounting Medium from Sigma-Aldrich® Chemical Co. (St. Louis, MO, USA) between the glasses. This solution keeps the fluorescence more time by stabilizing fluorophores, preventing fading and dryness of the sample. Without Fluoromount™ the fluorescence remains approximately one or two days with Alexa Fluor® dyes (a very stable fluorophores), but with Mounting Medium and keep at 4ºC, after 2 weeks its possible see fluorescence signal.

2.2.3. Confocal Microscopy

Confocal microscopy offers several advantages over conventional optical microscopy, including controllable depth of field, the elimination of image degrading out-of-focus information, and the ability to collect serial optical sections from thick specimens. The key to the confocal approach is the use of spatial filtering to eliminate out-of-focus light or flare in specimens that are thicker than the plane of focus. The confocal approach has facilitated much more useful imaging of living specimens, enabling the automated collection of three-dimensional (z-series) data, and improving the images obtained of specimens using multiple labeling. The point of light was focused by an objective lens at the desired focal plane in the specimen, and light that passed through it was focused by a second objective lens at a second pinhole having the same focus as the first pinhole. Any light that passed the second pinhole struck a low-noise photomultiplier, which generated a signal that was related to the brightness of the light from the specimen. The second pinhole prevented light originating from above or below the plane of focus in the specimen from reaching the photomultiplier (Paddock S.W. et al., 2013). This equipment, developed by Marvin Minsky in 1955 is proving to be one of the most important advances already achieved in optical microscopy (Amos and White, 2003).

Our images ware made in Electron Microscopy Center at Federal University of Rio Grande do Sul, in a *laser* scanning confocal microscope Olympus FluoView™ 1000 (Olympus® Inc. Tokyo, Japan). The image acquisition software and image processing was made in FluoView® (Olympus® Inc. Tokyo, Japan).

3. RESULTS

3.1. Evaluation of best fixative solution

Paraformaldehyde and methanol:acetone were tested in differentiated SH-SY5Y cells line to evaluate the presence of neurites, as show in Figure 1. Some scanning electron microscope (SEM) images were made by our group and were used to compare the proliferative and differentiated cells morphology. It is possible to see long cellular process in SEM images (Fig 1A

and 1B), as well as in phase contrast images in culture plate without any fixation (Fig 1C). This profile was compare to confocal images (visible channel) in Figure 1C and 1D.

3.2. The choice of the antibodies

Each primary antibody was tested in differentiated SH-SY5Y cells line each primary antibody in three different concentrations, in conventional fluorescence microscopy. The mouse anti-cytochrome c in 20 µg/mL, 10 µg/mL and 5 µg/mL; rabbit anti-cofilin-1 in 1 µg/mL, 0,5 µg/mL and 0,1 µg/mL. The secondary antibodies were tested in different dilutions (1:100 and 1:500) as shown in Figure 2. Based on the results, we established the best concentrations being 10 µg/mL to anti-cytochrome c, 0,5 µg/mL to anti-cofilin-1 and secondary antibodies 1:250 in buffer.

In cofilin-1 panel, it is possible to observe in the same concentration of primary antibody, differences between Alexa Fluor® 488 and Alexa Fluor® 635. Alexa Fluor® 488 labeled all cell including the neurites, as related in literature, but Alexa Fluor® 635 just label in the cell soma.

In cofilin-1 panel we highlight one cell to show distribution of cofilin in the cell cytoplasm, in Figure 3. It is possible see the presence of cofilin in all cytoplasm and in neurites, especially in some protrusions.

3.3. Mitochondrial fragmentation in response to 6-hidroxidopamenine treatment

6-OHDA treatment induces loss of mitochondrial membrane potential in proliferative and differentiated SH-SY5Y cells (Lopes F.M. et al., 2010). To evaluate this effect by microscopy, differentiated SH-SY5Y cells were treated with 15 µM 6-OHDA for 3h and 6h and immunofluorescence protocol was performed. The cells were labeled with anti-cytochrome c, Alexa Fluor® 568 and Hoechst® 33258. The confocal microscopy images were obtained as described in materials and methods and show in Figure 4.

In untreated SH-SY5Y cells is possible to see the classical mitochondria network, (Fig 4A), but when the cells are treated for 3h with neurotoxin, the

mitochondrial network appears to be undone and become more rounded (Fig 4B). After 6h treatment the mitochondria is completely fragmented, as shown in Fig 4C. The neurites are also affected. In non-treated cells, the neurites are labeled by cytochrome c (Fig 4D), but in 6h-treatment are unlabeled, as seen in Figure 4E.

4. DISCUSSION

Dephosphorylation (activation) of cofilin-1, an actin binding protein, is stimulated by initiators of neuronal dysfunction and degeneration including oxidative stress, excitotoxic glutamate, ischemia, soluble forms of beta-amyloid peptide, and its oxidation mediated neuronal cell death when it is induced by neurotoxins (Bamburg et al., 2010). Some studies established several advantages of the use of differentiated human neuroblastoma SH-SY5Y cells as an *in vitro* experimental model for physiopathology of Parkinson´s disease (Xie et al., 2010), enabling better understanding on molecular mechanisms that underlies neurodegeneration of the nigrostriatal pathway during the progression of Parkinson´s disease. 6-hydroxydopamine destroys dopaminergic neurons through free radical–mediated mechanisms, thus reproducing an important pathological feature of PD (Jenner, 2003; Schapira AH, 2011). To study cellular changes the confocal technology is proving to be one of the most important advances already achieved in optical microscopy (Amos and White, 2003). One of the most advantageous features of the *in vitro* model of DP when we work with microscopy is the morphology. These cells easily change its morphology in response of many kinds of injury and are very sensitive. Because of this, it is important to choose a fixative that do not change the cells characteristics, and enables to evaluate the modifications caused by 6-OHDA treatment. We test some fixatives, but we did not find differences between paraformaldehyde and methanol:acetone fixation. The both fixatives maintained the neurites well done. Thus, paraformaldehyde was chosen as fixative in our protocol, because it is better describe its reactions with other chemical compounds.

In an immunofluorescence protocol, is essential test the all antibodies available. In cofilin-1 panel, it is possible observe in the same concentration

of primary antibody, differences between Alexa Fluor® 488 and Alexa Fluor® 635. We attribute this effect to a possible differences between lots of antibody considering that are proteins derived from animals, even undergoing a internal rigid quality control by manufacturer, some differences are inherent of the production process of F(ab')2 Fragment (recognition and antigen-binding antibody region). Thereby, we reaffirm the need to test all antibodies combinations before realize the experiments. Another aspect also observed for the choice of the secondary antibody was the influence of color on the resolution limit, as mentioned in materials and methods of this article. We have chosen the more sensitive probe available to value translocation of cofilin-1 for mitochondria, thereby facilitating the assessment of intracellular mobilization of this protein. Thus, Alexa Fluor® 488 was chosen as secondary antibody to label cofilin-1. Curiously, we observed a clear difference between concentrations 1:100 and 1:500 in Alexa Fluor® 405 in cytochrome c panel. This secondary antibody in high concentrations accumulates in nucleus. Probably this effect is due some affinity of fluorochrome to binds in the DNA in high concentrations.

Considering our aim is develop an immunofluorescence protocol of detects cofilin-1, we have been successful. In Figure 3 is possible see a differentiated SH-SY5Y cell labels by cofilin-1 in all cytoplasm. The presence of cofilin-1 into the nucleus is reported by translocation in response to certain physical or chemical stimuli through an active nuclear localization signal (Munsie et al., 2012). What confirm our image, this protein is practically ubiquitous in the cell, which indicates the importance of this protein in cell physiology and highlights its multifunctional character. This way it was possible to standardize a protocol, focusing on cofilin-1, allowing studies with 6-OHDA to assess the behavior of cofilin in cell.

A small part of SH-SY5Y cells not are responsive to the differentiation, showing a different phenotype, with a large cytoplasm, a big nucleus and more adherent to the plate or coverslip. In the larger cell was possible see with more definition the classical mitochondria network, as well as the mitochondrial fragmentation. Mitochondria are remarkably dynamic organelles that migrate, divide and fuse in response a biochemical process, such as 6-OHDA challenge that causes reactive oxygen species inducing

mitochondrial fragmentation. We can then observe in the differentiated cell the presence of mitochondria in filaments form along the neurites and known to programmed cell death is an important process in both health and disease. Although mitochondrial fission is an early event in cell death, the precise role of fission in cell death remains unclear. Each neuron contains several hundred mitochondria that form cable-like structures along neuronal projections to help them meet their large energy demands. Neurons require energy to transport organelles and cargo along microtubules or actin fibres (motor molecules like dyneins, kinesins and myosin mediate this process) and to maintain ion gradients and the membrane potential by ATP-dependent Ca^{2+} and Na^{+}/K^{+} pumps and ion channels. Additionally, neurotransmitter vesicle loading at pre-synaptic terminals and Ca^{2+}-mediated neurotransmitter release into the synaptic cleft are also ATP-dependent events (Knott et al., 2008). This explain the presence of cytochrome c in neurites healthy cells in figure 4C, but not in cells treated with 6-OHDA. Although is important to emphasize, cytochrome c is describe as mitochondrial marker and its role in oxidative phosphorylation is very establish, it is not the best mitochondrial marker, because it is easy to oxidize and release from mitochondria, however the images with MitoTracker® are unclearly and was not be used for any biological evaluation in this work.

Confocal microscopy does not make miracles, makes possible visualized same structures with greater definition, however other structures, perhaps little noted in tradition fluorescence microscopy become a big problem in confocal microscopy such as for example, crystals formed by salts or aminoacids. Unfortunately, because of such problems as microscopy artifacts we not be able to value the behavior of cofilin-1 when the cell are treated by 6-OHDA, thus is necessary more test, because this protein is reported as can be a target for therapeutic studies.

Howsoever, with our results, we can conclude that the protocol developed is useful for biochemical and cytomorphologic changes evaluations induced by the neurotoxin 6-hydroxydopamine in SH-SY5H cells line.

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FIGURES

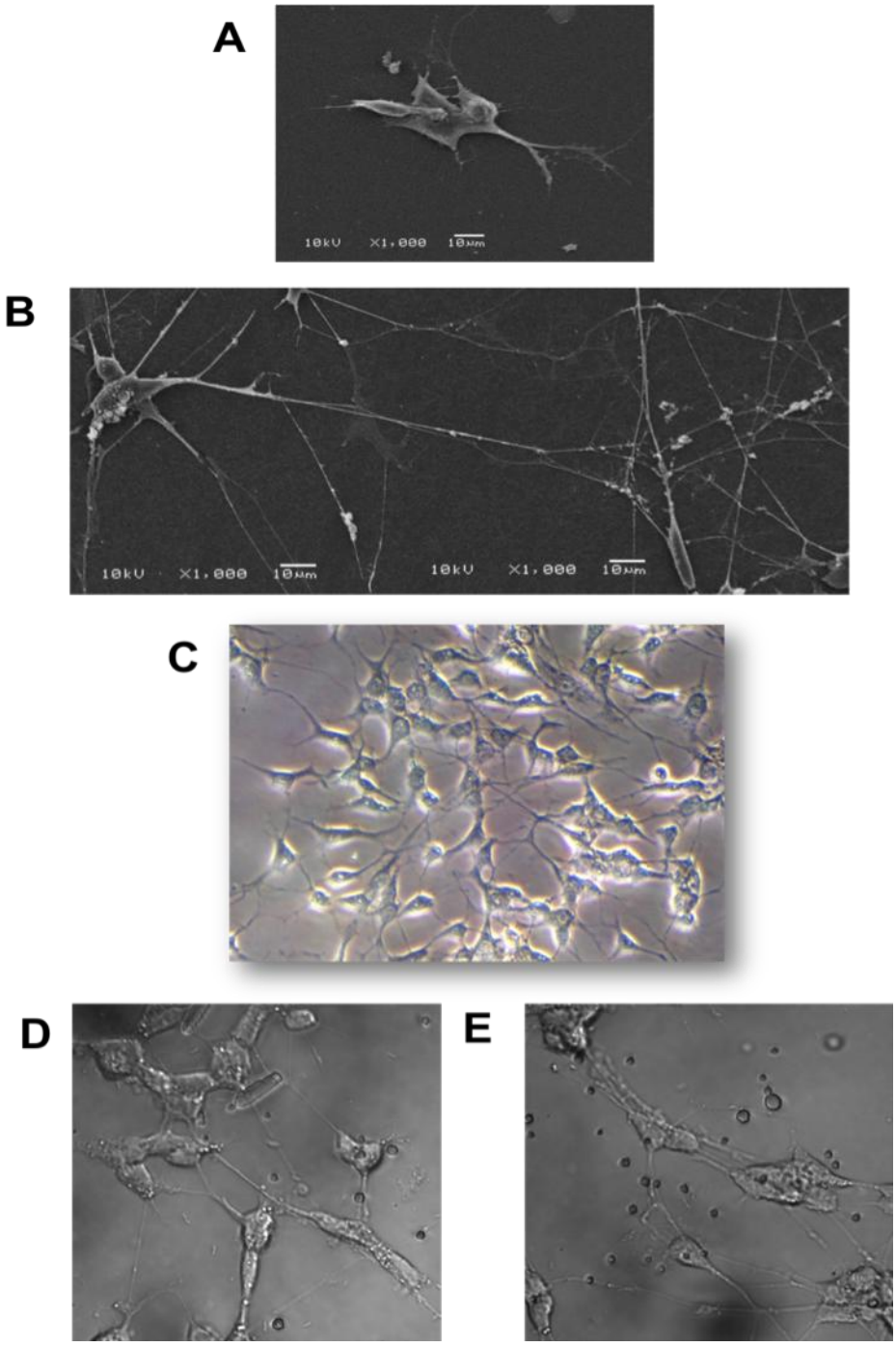
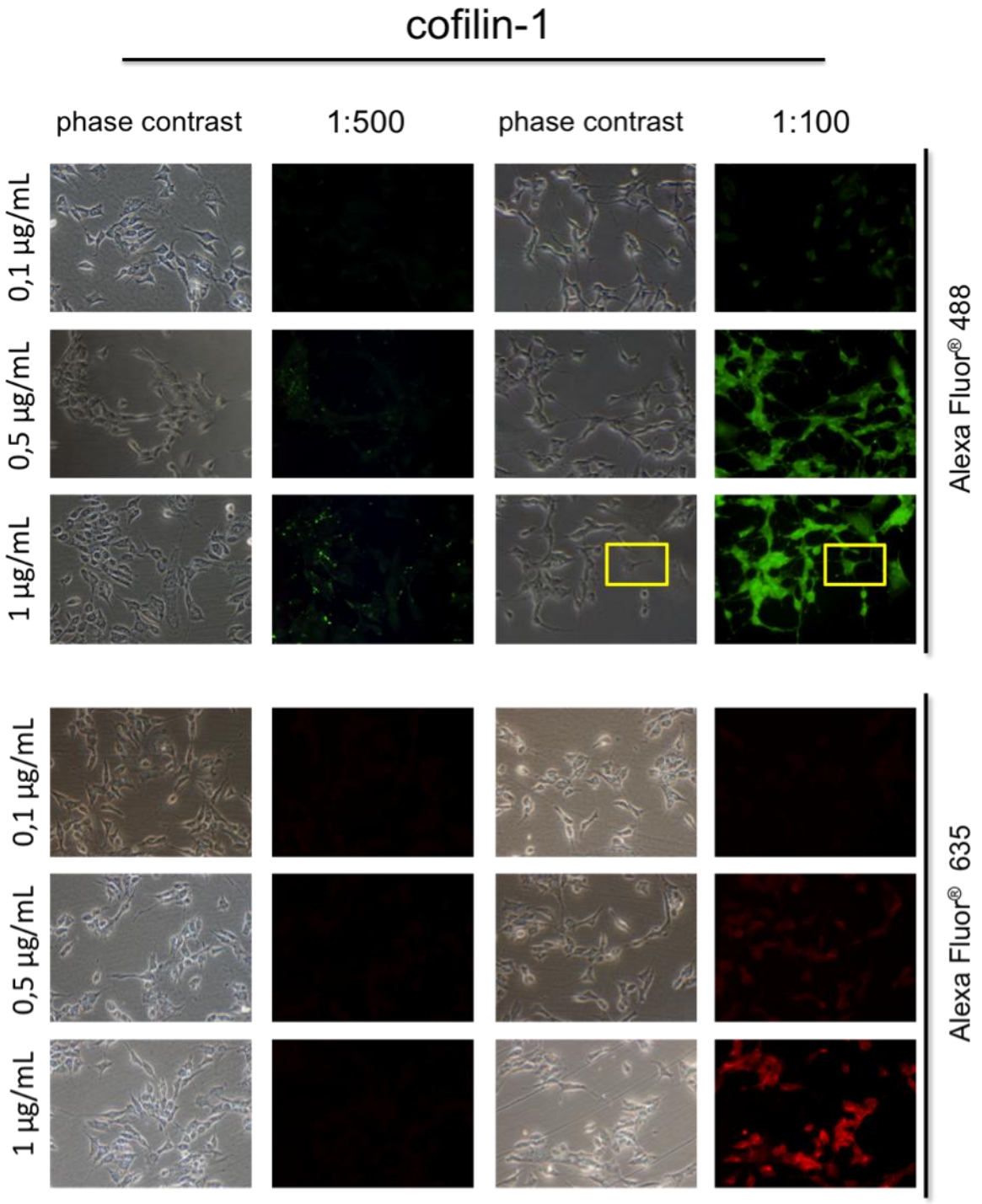


Figure 1. A) Scanning electron microscope (SEM) image of proliferative SH-SY5Y cells line showing short neurites. B) SEM image of differentiated SH-SY5Y cells line with long neurites. Magnification 1.000x. C) Phase contrast image of cells without fixation in culture plate. Magnification 200x. D) Proliferative SH-SY5Y cells line fixed by methanol:acetone (1:1 v/v). E) SH-SY5Y cells fixed by paraformaldehyde 4% (m/v). Confocal Microscope, magnification 400x.

A


cofilin-1
phase contrast
1:500
phase contrast
1:100
0,1 µg/mL
0,5 µg/mL
1 µg/mL
Alexa Fluor® 488
0,1 µg/mL
0,5 µg/mL
1 µg/mL
Alexa Fluor® 635

B

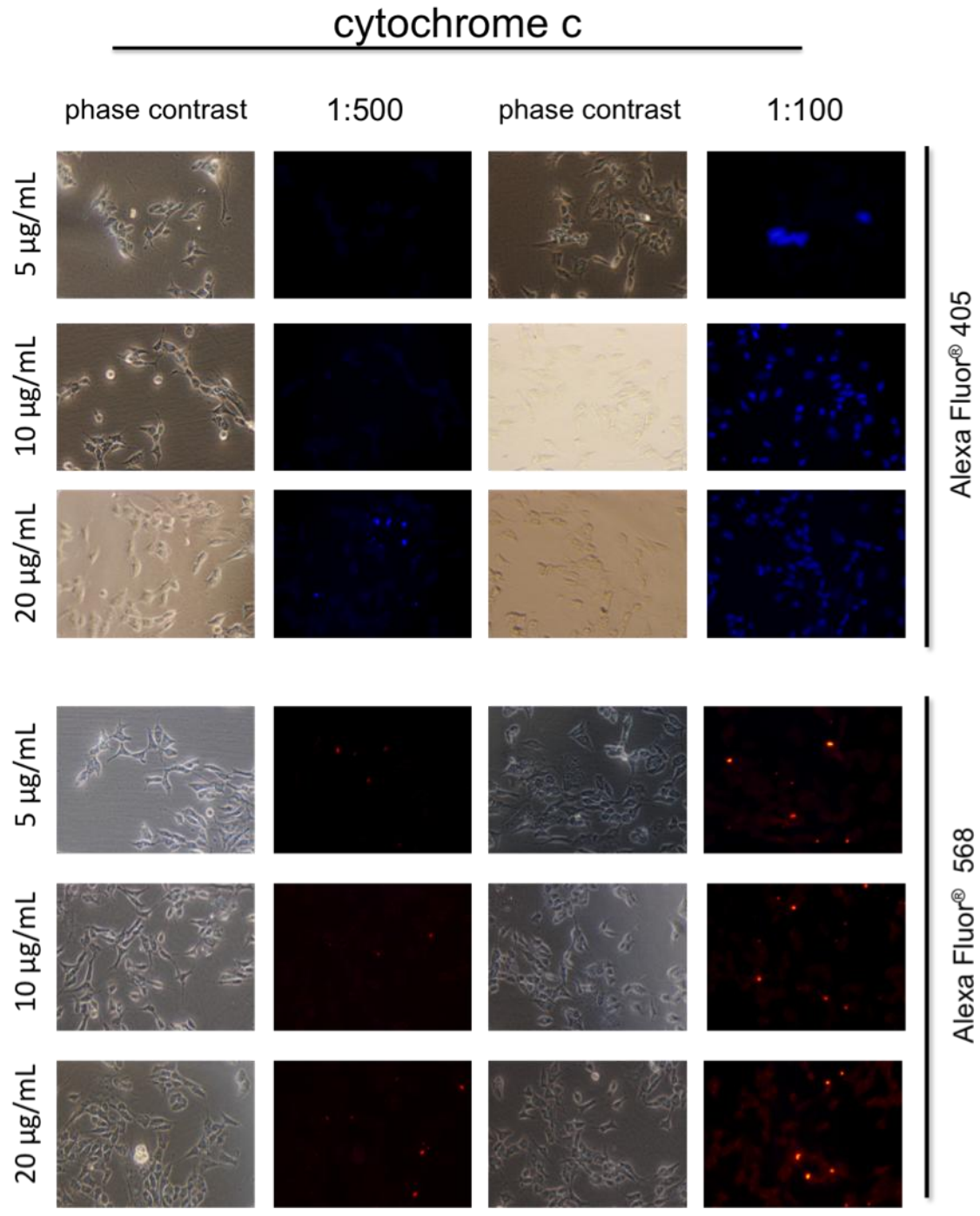
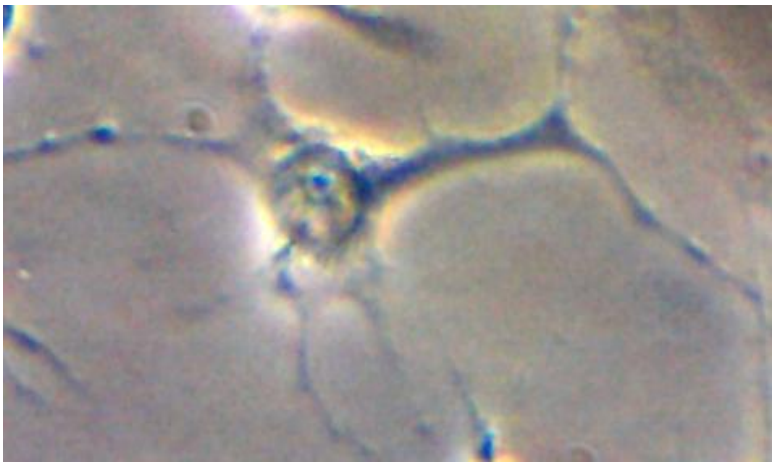


Figure 2A, 2B. Standardization antibodies panels show three concentration of primary antibody (rows) and two dilutions of secondary antibody (columns). Magnification 200x in conventional fluorescence microscope.

A



B

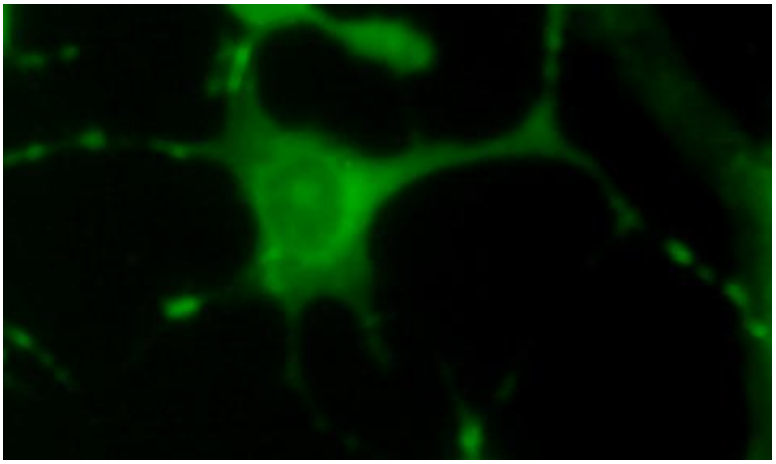


Figure 3A, 3B. Photomicrograph of differentiated SH-SY5Y cells line labeled with anti-cofilin-1 and anti-Alexa Fluor® 488, shows the presence of cofilina-1 all cytoplasm including neurites and nucleus (Magnification 200x).

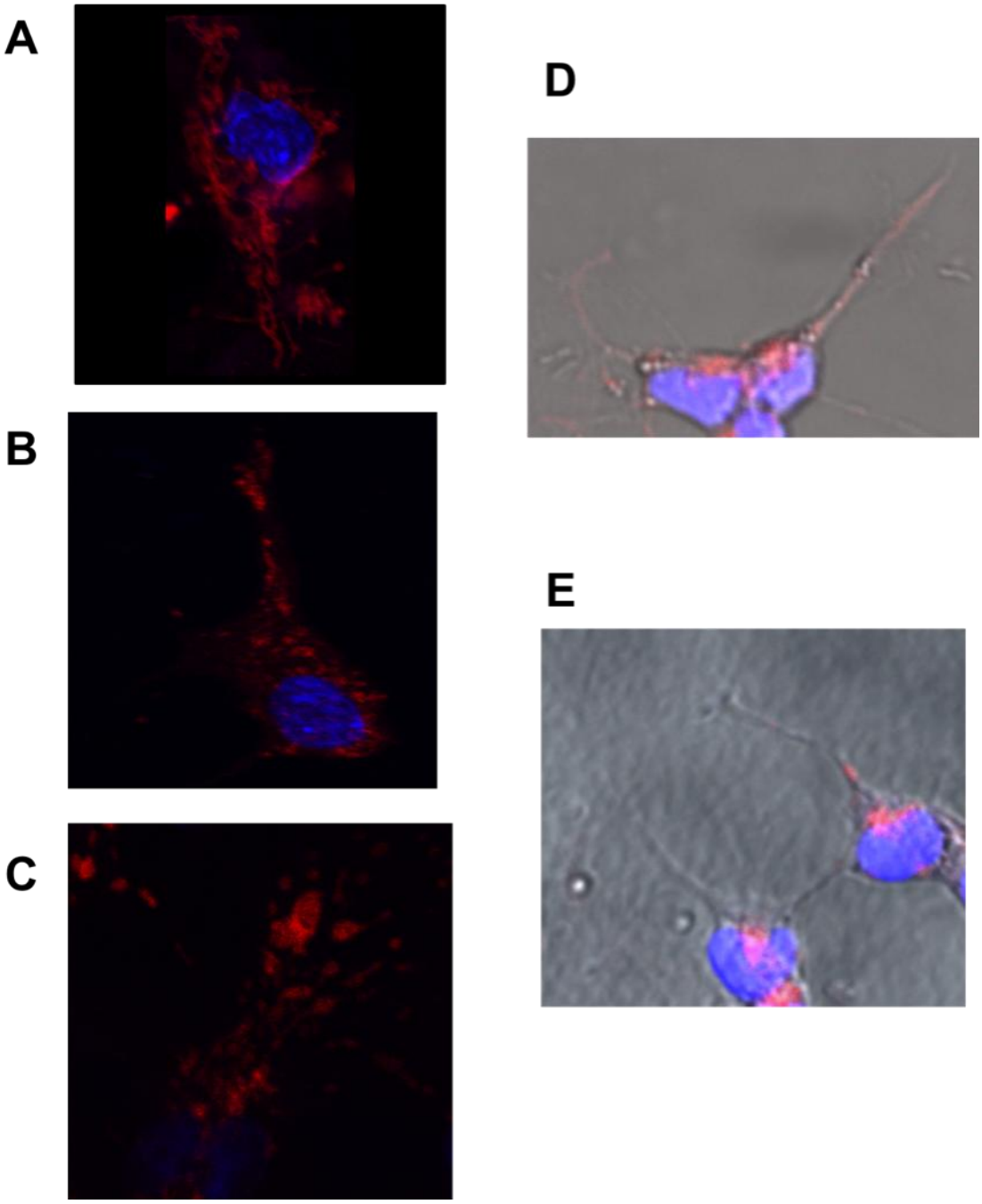


Figure 4. Differentiated SH-SY5Y cells line labeled with anti-cytochrome c, anti-Alexa Fluor® 568 and Hoechst® 33258. A) Cell without 6-OHDA treatment, shows a classical mitochondria network. B) SH-SY5Y cells treated with 6-OHDA for 3h, shows early mitochondrial disruption. C) Cells treated with 6-OHDA for 6h, exhibiting mitochondrial fragmentation (400x magnification). D) Differentiated SH-SY5Y without 6-OHDA treatment presents neurites labeled by cytochrome c indicating the presence of mitochondrias into the neurites. E) Differentiated SH-SY5Y treated with 6-OHDA for 6h shows neurites with loss of labeling of cytochrome c (Magnification 400x).

3 CONCLUSÕES E PESPECTIVAS

Um dos grandes desafios no estudo sobre os processos fisiopatológicos é compreender como ocorrem determinados acontecimentos celulares em diversas doenças. Várias doenças neurodegenerativas, incluindo a DP, tem como base fisiopatológica a disfunção mitocondrial, o estresse oxidativo e a agregação protéica. No entanto, os mecanismos moleculares que levam a estes processos ainda devem ser elucidados [20]. Contudo um dos maiores impeditivos são as próprias técnicas utilizadas para formular tais respostas, pois cada técnica possui suas limitações, condições não fisiológicas, reagentes, detergentes e outros que nos fornecem subsídios para formular respostas para questionamentos existentes, mas sempre levando em consideração as modificações exigidas para tal técnica. Para observar fenômenos celulares com o mínimo possível de interferência de técnica, a microscopia é uma das técnicas mais adequadas por ser descrita como menos invasiva, pois mantém a estrutura celular afim de que a célula seja observada tendo com interferentes os corantes e fluoróforos utilizados tal qual o momento em que foi fixada.

Apesar de citarmos a microscopia como uma técnica de pouca interferência metodológica, ainda devemos considerar o trabalhoso e longo processo ao qual as células são submetidas durante o protocolo de imunofluorêscencia. Este protocolo inclui inúmeras lavagens com tampões, detergentes que permeabilizam a membrana celular para permitir a passagem de outros reagentes, fixação - que estabiliza os componentes celulares, sejam eles proteicos, lipídicos e outros causando a morte da célula, morte esta que deve ser rápida para não induzir alterações experimentais. A utilização do equipamento também exige considerável atenção, pois conforme cada tipo de microscópio a amostra deve possuir uma determinada espessura máxima, estar sob certas superfícies, questões que nem sempre são fáceis de adaptar às necessidades da amostra biológica. Nem todo tipo celular possui as mesmas características. Vários tipos celulares são cultivados em suspensão, não aderindo à superfície, e

algumas células só aderem a certos subtratos após tratamento com resinas ou polímeros. Com tantas variáveis, este trabalho buscou relatar pontos a serem considerados ao escolher a microscopia, em especial a microscopia confocal, para que outros novos pesquisadores possam adaptar seus espécies a serem observados. Em consequência da falta de adaptação dos protocolos as amostras, podemos observar uma subutilização dos equipamentos disponíveis. Como descrevemos, a utilização da microscopia não está limitada a apenas a imunofluorescência, mas às diversas outras técnicas que utilizam o princípio de excitação e emissão de sinal por compostos fluorescentes. Assim, devemos explorar ainda mais os potencias destas técnicas.

A linhagem celular escolhida para este trabalho é extremamente sensível, então todos os cuidados foram tomados a fim de que suas estruturas fossem preservadas para que as avaliações pudessem ser atribuídas a efeitos bioquímicos e não a interferências metodológicas, e obtivemos sucesso. Forma testados alguns fixadores, como paraformaldeído e metanol:acetona, entretanto não encontramos diferenças entre estes fixadores. Os dois fixadores mantiveram bem a morfologia dos neuritos. Assim, o paraformaldeído foi escolhido como fixador no nosso protocolo porque está melhor descrito suas reações com outros compostos químicos, fato que deve ser observado. Ser o mais utilizado não significa ser o melhor reagente, mas utilizar um reagente amplamente conhecido facilita a busca e troca de informações.

Um dos objetivos deste trabalho, a avaliação do comportamento de cofilina-1 em resposta ao tratamento de 6-OHDA, infelizmente foi parcialmente alcançado. Ao que se refere à padronização do protocolo de marcação de cofilina-1, obtivemos êxito, pois as proteínas foram marcadas e apresentaram distribuição citoplasmática e nuclear conforme a literatura descreve. Contudo, mesmo com a padronização de cada etapa do protocolo, os artefatos de microscopia são frequentes nas preparações. Para a obtenção de boas imagens, devemos ter atenção com cada etapa da preparação, pois uma única etapa realizada com descuido pode colocar em risco todo o experimento, pois alguns erros cometidos no início do protocolo só são visualizados no final. Dessa forma, todos os passos desde a lavagem

correta das lâminas e lamínulas utilizadas, o correto plaqueamento das células, e controles de reagentes são fundamentais. Apesar de estarem disponíveis softwares de melhoramento de imagem, o cuidadoso preparo da amostra e a padronização de técnicas de aquisição de imagens sempre é o ideal. Ainda assim, a utilização de softwares de processamento de imagem é um recurso eficaz para a obtenção de informações que sem os mesmos seriam perdidas devido aos efeitos ópticos ou problemas no preparo da amostra [66]. Assim, um dos questionamentos feitos durante este trabalho acerca da mobilização de cofilina-1, tendo em vista que esta proteína é amplamente difundida na célula e está relacionada a diversas funções, refere-se a qual seria a quantidade mínima de cofilina-1 a ser mobilizada para a mitocôndria para que esta seja detectável? Por western blot, esta mobilização pode ser quantificada, entretanto devemos considerar a sensibilidade das quantificações feitas a partir de imagens obtidas por microscopia confocal para determinar esta mobilização. Dessa forma, são necessários mais testes para verificar qual a quantidade de sinal mínima é necessária para avaliar se a mobilização de cofilina-1 é quantificável.

MitoTracker® é um corante catiônico lipofílico, que torna-se fluorescente por reações de oxidação dentro da mitocôndria, onde ocorre ligação de seus grupamentos tióis em proteínas mitocondriais [67, 68], no entanto, quando em concentrações maiores o corante se torna inespecífico. Neste trabalho, a utilização deste reagente foi proposta como marcador mitocondrial, contudo, sua utilização foi impossibilitada, devido à ligação do corante a superfície de vidro e a marcação do núcleo, revelando problemas no reagente ou na forma como foi utilizado, podendo a fixação e a concentração utilizada não terem sido adequadas. Este é um corente bastante utilizado, sendo descrito em inúmeros trabalhos e se deve buscar padronizá-lo melhor na linhagem que utilizamos. Uma possível solução para este problema pode ser a utilização de poli-L-lisina para revestir a lamínula, mesmo quando as células não necessitem de poli-L-lisina para adesão, este reagente pode ajudar a bloquear a autofluorescência natural do vidro e impede que a presença de gorduras.

A mitocôndria, entre as organelas da célula animal, está caracterizada como uma das mais complexas. Tendo uma origem diferenciada das demais e sendo a única organela que possui DNA próprio, a mitocôndria exerce papel ímpar para a célula por estar envolvida com processos da respiração celular, apoptose e maior fonte de produção de espécies reativas. Neste trabalho, a dinâmica mitocondrial teve especial importância para a validação do protocolo como técnica com potencial de elucidar eventos bioquímicos.

Através da fragmentação mitocondrial, uma das consequências da formação de espécies reativas e uma das características da apoptose, há a fissão mitocondrial em que as mitocôndrias não estão mais conectadas umas às outras. Esse evento tem consequência relevante em células neuronais, pois cada neurônio contém centenas de mitocôndrias que formam estruturas semelhantes a um cabo ao longo de projeções neuronais, para ajudá-los a satisfazer as suas grandes demandas de energia, como foi possível visualizar na Figura 4D do artigo. Os neurônios necessitam de energia para o transporte de organelas e de carga ao longo de microtúbulos ou fibras de actina (moléculas motoras como dineínas, cinesinas e miosina para mediar este processo) e para manter os gradientes de íons e o potencial de membrana dependente Ca^{2+}, bombas Na^{+}/K^{+} e canais iônicos. Além disso, a liberação de vesícula com neurotransmissores nos terminais pré-sinápticos e a liberação de Ca^{2+} na fenda sináptica também são eventos dependentes de ATP [69], como é possível ver na Figura 4.

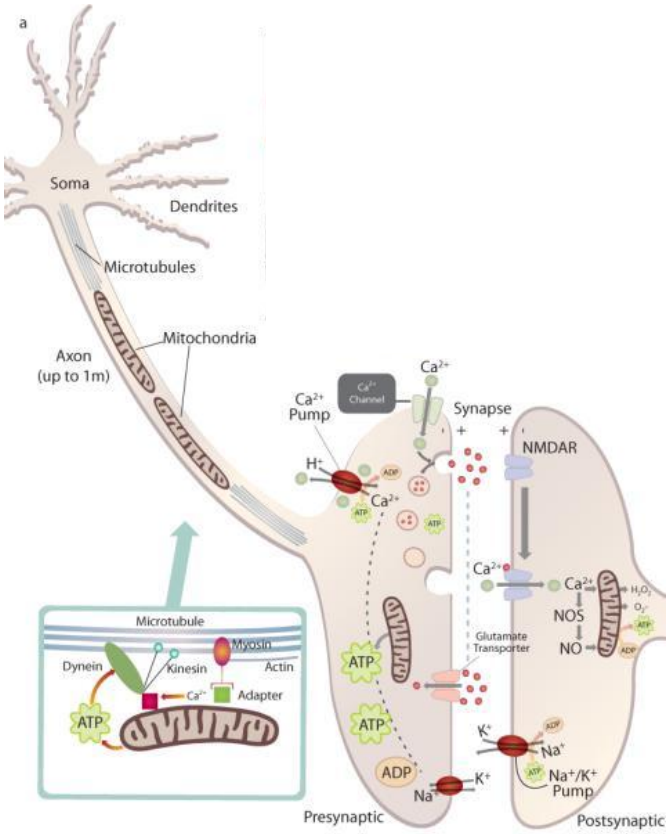


Figura 4: Representação da presença de mitocôndrias nos processos neuronais (Adaptado de Knott *et al*, 2008).

Assim, podemos concluir que a microscopia confocal possui diversas vantagens metodológicas que possibilitam avaliações citomorfológicas e bioquímicas da célula. Ainda, que a neurotoxina 6-OHDA induz fragmentação mitocondrial como descrito em processos de morte celular programada [29, 65] e que a cofilina-1 está amplamente difundida no citoplasma das células da linhagem SH-SY5Y diferenciada. Por fim, podemos reforçar as vantagens desta linhagem como modelo *in vitro* para estudos da Doença de Parkinson.

Este trabalho tem como perspectiva ampliar e potencializar o uso da microscopia de fluorescência, em especial, a microscopia confocal como ferramenta de estudo. Este trabalho deve ser continuado para que seja possível determinar por métodos de imagem a translocação de cofilina-1 para a mitocôndria na linhagem celular SH-SY5Y, corroborando com os dados da literatura. Este trabalho também deve servir de apoio técnico para

futuras avaliações através de microscopia para este tipo celular, não se limitando a imunofluorescência, mas tornando possível que métodos mais sofisticados, como a transfecção de proteínas repórteres e análises que visem à quantificação *in vitro* de eventos bioquímicos na célula viva sejam realizados.

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5 ANEXOS

5.1 Protocolo Desenvolvido

Protocolo de Imunofluorescência para Microscopia Confocal:

Etapas pré-protocolo de imunofluorescência

Preparo das lâminas e lamínulas

- Mergulhar as lamínulas circulares de vidro, 15mm ou 18mm, em água e detergente, e aquecer até ferver (no micro-ondas).
- Enxaguar com água corrente até sair totalmente o detergente.
- Mergulhar no álcool 70% e deixar overnight.
- Colocar as lamínulas para secar em estufa utilizando uma pinça. Não utilize nenhum tipo de papel para realizar a secagem.
- Deixar em luz UV por no mínimo 30min.
- Colocar na placa de 12 poços ou 24 poços.

Plaqueamento das células

- Tripsinizar as células SH-SY5Y em estado proliferativo e semear 22.500 cél/cm² para lamínulas de 15mm ou 40.000 cél/cm² para lamínulas de 18mm. (Quantidade referente a 10% menos que o descrito para manutenção do cultivo).
- Pipetar a suspensão de células em meio DEMEM/F12 + 10% Soro Feral Bovino (SFB) + 1% de antibiótico sobre área da lamínula, de forma que não extravaze para fora da área do vidro.
- Deixar por aproximadamente 1h em estufa a 37°C e 5% de CO_2.
- Completar com a quantidade necessária de meio para manutenção do cultivo na placa (para placa de 12 poços, 1 mL, para placa de 24 poços, 500µL).
- Deixar overnight na estufa 37°C e 5% de CO_2.

Protocolo de diferenciação celular

- Após adesão das células, o meio deve ser substituído por meio DMEM/F12 contendo 1% SFB e 1% antibiótico e adicionado 10µM de Ácido Retinóico.
- A substituição do meio deve ser feita a cada 3 dias, durante uma semana. Após este período as células devem apresentar morfologia estrelada e não haver células em divisão celular e poucas células em apoptose.

Tratamento com neurotoxina 6-hidroxidopamina

- Preparar Falcon com 10 mL de 1% de ácido ascórbico e diluir 0,01g de 6-hidroxidopamina (sigma) Concentração da solução será de 400µM de 6-OHDA.

- Dilua em meio DMEM/F12 +1% SFB e 1% antibiótico a quantidade necessária para obter uma concentração final de 15µM de 6-OHDA.
- Deixar a placa em estufa a 37°C e 5% de CO2 o tempo estabelecido para estudo.

Etapas do Protocolo de imunofluorescência

- Tirar meio das células
- Lavar as células 3x com Tampão Fosfato Salino (PBS) sem Phenol red.
- OBS: Todas as soluções até a etapa de fixação devem estar à 37°C.

Marcação com Mitotracker® Orange e Hoechst® 33258

- Adicionar da solução de estoque de Hoechst (5mg/mL) em PBS – Concentração final de uso: **1µg/mL**
- Adicionar da solução de estoque de Mitotracker (1mM em DMSO) em PBS – Concentração final de uso: **400nM**
- Deixar incubar por 30min a 37°C
- Lavar com PBS 1x.

Fixação Paraformaldeído

- Pré-fixar utilizando Paraformaldeído 4**%** em PBS (1:1 v/v) por 20min em temperatura ambiente. (concentração final de Paraformaldeído será de 2%)
- Desprezar fixadores.
- Fixar com Paraformaldeído **4%**, por 30min a temperatura ambiente.
- Lavar com PBS 3x deixando em agitação por 5min.

Permeabilização e Bloqueio

- Lavar 2x com PBS + 0,2 %Triton x-110, deixando em agitação por 5min.
- Lavar 2x com PBS + 0,2 %Triton x-110 + 1% BSA em agitação por 5min.
- Encubar por 1h com PBS + 1% BSA, 1mL, deixando sob agitação por 5min a 4°C.

Incubação Anticorpo Primário

- Diluir anticorpo primário em PBS + 1% BSA.
  - Citocromo C: **10µg** (estoque: 500µg/mL)
  - Cofilina: **0,5µg** (estoque: 500µg/mL)
- Colocar o anticorpo primário e deixar em câmera úmida, overnight a 4°C.
- Deixar a placa durante 30min à temperatura ambiente.
- Lavar as células 2x PBS + 0,2 % Triton X-110.
- Lavar as células 2x com PBS por 5min em agitação.

Incubação Anticorpo Secundário

- Diluir anticorpo secundário em PBS (1:250 v/v) em um eppendorf e distribuir nos poços segundo a combinação de origem dos anticorpos.
    - Alexa Fluor 488(Verde) – rabbit → cofilina
    - Alexa Fluor 568 (Vermelho) – mouse → citocromo c

- Encubar 1h a 4°C em agitação
- Lavar 2x com PBS + 0,2 %Triton x-110 em agitação por 5min.
- Lavar com PBS 2x deixando em agitação por 5min.

Selagem da Lamínula

- Sob uma lâmina limpa, pingue uma gota do meio de montagem Fluoromont.
- Retire do poço a lamínula, com cuidado e coloque-a sobre a gota de forma que o lado que as células fiquem entre o meio de montagem e a lâmina. Evite a formação de bolhas entre a lâmina e a lamínula.
- Seque o excesso de meio de montagem com auxilio de um papel ou espontaneamente s por aproximadamente 15 min.
- Sele a lamínula com esmalte, preenchendo toda a borda da lamínula. Não passe esmalte por cima da lamínula, apenas nas bordas.
- Deixe secar completamente.
- As lâminas estão prontas para serem observadas.

5.2 Normas para apresentação oral e escrita do trabalho experimental do estágio em pesquisa e monografia.

1. O trabalho experimental do aluno, realizado obrigatoriamente durante a atividade Estágio em Pesquisa, deverá ser apresentado na forma escrita e oral ao final dos seis meses da atividade.

2. Aquele aluno que optar por realizar o Estágio Curricular Supervisionado em Biomedicina em atividade de pesquisa, sob a orientação do mesmo Professor/Pesquisador e na mesma linha de pesquisa do Estágio em Pesquisa e Monografia, poderá apresentar o seu trabalho ao final dos seis meses deste segundo estágio (neste caso o aluno, por não ter realizado o estágio supervisionado, recebe apenas a habilitação conferida pelo estágio de pesquisa).

Do trabalho escrito:

1. O trabalho escrito deverá ser organizado como proposto a seguir.

a. Folha de rosto com título, nome do aluno, nome do orientador, nome do coorientador quando existente, curso e ano.

b. Agradecimentos e dedicatória (quando existentes).

c. Índice geral.

d. Resumo.

e. Introdução compreensiva.

f. Trabalho experimental na forma de artigo científico, seguindo a formatação exigida pelo periódico onde seria submetido, ainda que os resultados obtidos sejam apenas preliminares.

g. Conclusões e Perspectivas.

h. Bibliografia adicional que não esteja presente no artigo científico.

i. Anexos (quando existirem).

2. O aluno poderá optar por escrever o artigo científico, referido no item (f) acima, na língua inglesa.

3. O trabalho deverá ser impresso em folha A4, com tipo de letra tamanho 12, considerando como página 'um' a folha de rosto e respeitando as seguintes margens:

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b. Margem direita: 2,5 cm;
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d. Margem inferior: 2,5 cm.

4. O aluno deverá entregar uma cópia impressa do trabalho escrito para cada membro da Banca Examinadora pelo menos 15 dias antes da apresentação oral. Junto a esta cópia deve ser entregue uma cópia das orientações da COMGRAD-BIM aos examinadores das bancas de TCC.

5. Antes da apresentação o aluno deverá informar à Comissão de Graduação os dados da monografia (título, resumo), nomes dos membros da banca e de co-orientador e a titulação destes profissionais para fins de confecção dos certificados.

6. O aluno deverá entregar à Comissão de Graduação uma cópia impressa e uma na forma eletrônica, em CD, do trabalho escrito, após terem sido efetuadas as correções solicitadas pela Banca Examinadora.

Da apresentação oral:

1. O aluno deverá apresentar oralmente o seu trabalho no dia e horário determinados pela Comissão de Graduação para este fim.
2. Será alocado a cada aluno 20 minutos para a apresentação oral e 10 minutos adicionais para arguição dos membros da Banca Examinadora.

Da Banca Examinadora:

1. A Banca Examinadora será constituída de dois professores, pesquisadores ou doutorandos indicados pelo orientador do aluno.
2. Não será exigida presença obrigatória dos integrantes da Banca Examinadora no dia da apresentação oral, mas a participação destes é estimulada.

Do conceito final:

1. O conceito final do aluno será calculado a partir dos conceitos emitidos pelos integrantes da Banca Examinadora e do orientador do aluno.
2. O orientador também deverá emitir um conceito pelo Estágio de Pesquisa.

5.3 Formatação da revista científica – Journal of Neuroscience Methods

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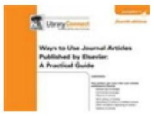

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