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SYSTEMATIC REVIEW ARTICLE

Using Cell Cultures for the Investigation of Treatments for Attention Deficit Hyperactivity Disorder: A Systematic Review

Danielly Chierrito[1], Camila B. Villas-Boas[2], Fernanda S. Tonin[3], Fernando Fernandez-Llimos[4], Andréia C.C. Sanches[2] and João C.P. de Mello[1,*]

[1]*Department of Pharmacy, Universidade Estadual de Maringá, Maringá, PR, Brazil;* [2]*Department of Medical and Pharmaceutical Sciences, Universidade Estadual do Oeste do Paraná, Cascavel, PR, Brazil;* [3]*Department of Pharmacy, Universidade Federal do Paraná, Curitiba, PR, Brazil;* [4]*iMed.ULisboa, Department of Social Pharmacy, Faculty of Pharmacy, Universidade de Lisboa, Lisboa, Portugal*

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Abstract: ***Background*:** Advances in basic and molecular biology have promoted the use of cell cultures in a wide range of areas, including the evaluation of drug efficacy, safety and toxicity.

***Objective*:** This article aims to provide a general overview of the methodological parameters of cell cultures used to investigate therapeutic options for Attention Deficit Hyperactivity Disorder.

***Method*:** A systematic search was performed in the electronic databases PubMed, Scopus, and DOAJ. *In vitro* experimental studies using cell cultures were included.

***Results*:** A total of 328 studies were initially identified, with 16 included for qualitative synthesis. Seven studies used neuronal cells (SH-SY5Y neuroblastoma and PC12 cell line) and nine used non-neuronal cells. All the studies described the culture conditions, but most studies were inconsistent with regard to reporting results and raw data. Only one-third of the studies performed cell viability assays, while a further 30% conducted gene expression analysis. Other additional tests included electrophysiological evaluation and transporter activity. More than 50% of the studies evaluated the effects of drugs such as methylphenidate and atomoxetine, while plant extracts were assessed in four studies and polyunsaturated fatty acids in one.

***Conclusion*:** We suggested a flowchart to guide the planning and execution of studies, and a checklist to be completed by authors to allow the standardized reporting of results. This may guide the elaboration of laboratory protocols and further *in vitro* studies.

Keywords: Cell model, neuronal cell, ADHD, ADHD treatment, methodological aspect, flowchart, checklist.

1. INTRODUCTION

Advances in basic and molecular biology have promoted the use of cell cultures in a wide range of areas, including for the evaluation of drug efficacy, safety and toxicity as well as for the production of vaccines and biopharmaceuticals [1]. This methodology allows the use of immortalized cell lines, primary cell cultures and stem cells-derived cell models in experiments, according to the research objective [2].

In the field of Attention Deficit Hyperactivity Disorder (ADHD), a neurological disorder that affects up to 7.2% of children and adolescents and 2.5% of adults [3-6], cell cultures have been used to investigate new therapeutic alternatives or to test the safety and toxicity of the available drugs [7-10]. Current therapeutic alternatives include psychostimulant drugs (methylphenidate, dexamphetamine), noradrenergic agents (atomoxetine), antidepressants and others [11, 12].

The most commonly used cell lines to investigate ADHD include neuronal cell lines (neuroblastoma SH-SY5Y) and non-neuronal cell lines such as HEK293rtTA, HEK-293, TsA201 and JAR, cells isolated from patients, and cells isolated from animals [9, 10, 13-21]. However, the effective use of cell cultures requires some fundamental aspects to be met. These include knowledge of the culture conditions (appropriate growth medium, micronutrients, temperature and pH), cell differentiation methods to allow the induction of neuronal dopaminergic and cholinergic phenotypes, cell viability methods to demonstrate cell survival or death in response to exposure to substances, and the evaluation of gene expression for possible cellular reactions due to pharmacological treatments [22-25]. The optimization and standardization of these parameters are essential to develop laboratory proto-

*Address correspondence to this author at the Department of Pharmacy, Universidade Estadual de Maringá, Maringá, PR, Brazil; Tel/Fax: +55 44 30114627; E-mail: mello@uem.br

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cols, in order to obtain higher quality results and guide further *in vitro* and *in vivo* research.

Currently, only a few studies exist about the standardization of cell culture models in the neurological field. Some recent systematic reviews have synthesized information on *in vitro* research for Parkinson's disease and Bipolar Disorder, but there is still no synthesis for cell culture models in ADHD [26, 27]. Thus, our objective was to perform a systematic review to provide a broader overview of the methodological aspects to the use of cell cultures in the investigation of therapeutic options to ADHD.

2. METHODS

This research was designed according to the recommendations from the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [28], Cochrane Handbook for Systematic Reviews of Interventions [29], and the Joana Briggs Institute [30]. The steps of screening article titles and abstracts, full-text reading, and data extraction were conducted by two independent reviewers, with a third reviewer consulted in case of discrepancies.

2.1. Systematic Literature Search and Eligibility

A systematic search was performed in the electronic databases PubMed, Scopus, and Directory of Open Access Journals (DOAJ) (September 2017). A manual search was also conducted of the bibliographic references of the included articles.

The inclusion criteria were *in vitro* experimental studies that used cell culture (any type of cell line) to investigate possible therapeutic options (any form, dosage or regimen) for the treatment of ADHD. Other types of studies (*e.g*. only *in vivo* evaluations, studies with humans), and studies not reporting data on cell culture or ADHD were excluded.

2.2. Data Extraction and Reporting Evaluation

A standardized form was used to extract data on the culture conditions and methodological aspects, the differentiation process, cell viability and proliferation methods, other additional tests of gene expression, electrophysiological evaluation and transporter activity.

Additionally, to better visualize the information provided by the studies on the methods used and the result reported, we applied an adapted version of the SYstematic Review Center for Laboratory Animal Experimentation tool (SYRCLE's) to the included studies [31].

3. RESULTS

A total of 328 studies were screened (titles and abstracts were read), after duplicate removal, of which 83 were included for full-text appraisal. Finally, 16 studies were eligible for qualitative synthesis, as shown in Fig. (**1**) [7-10, 13-21, 32-34].

The main characteristics of the included studies are presented in Table **1**. Studies were conducted in different countries, mostly in Germany (50%), and published between 2007 and 2017. Neuronal cell lines (neuroblastoma SH-SY5Y and PC12) and non-neuronal cell lines such as HEK293rtTA, HEK-293, TsA201, and JAR, cells isolated from patients such as human lymphocytes, lymphoblastoid

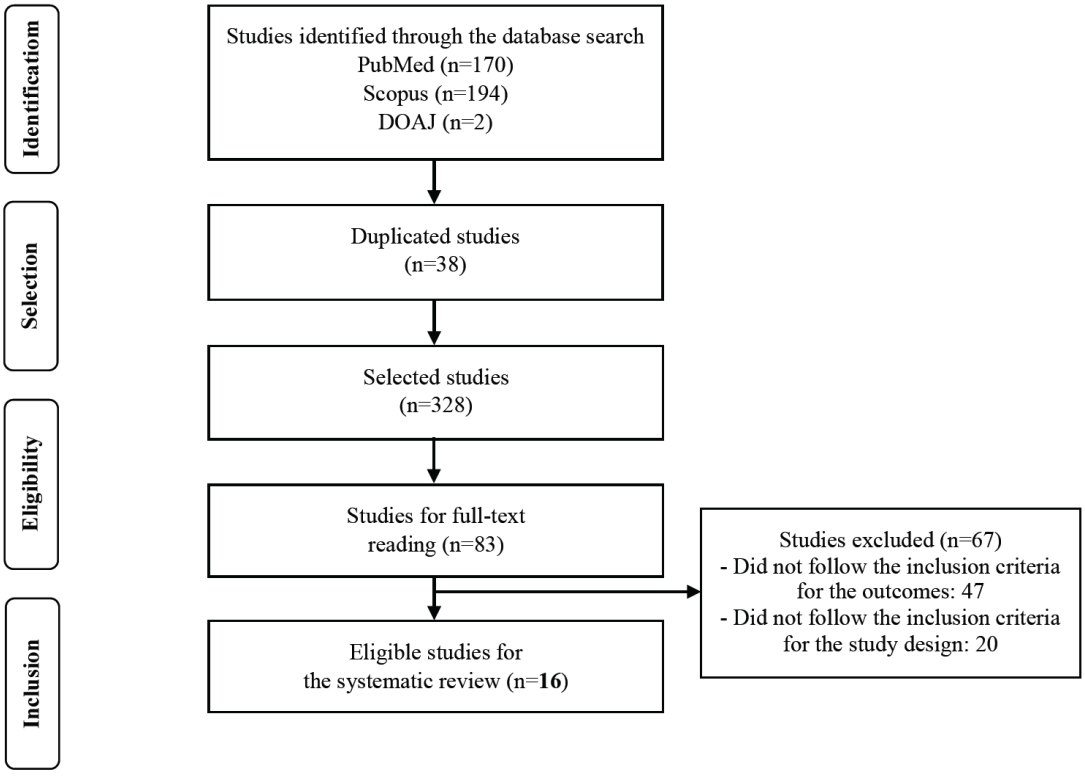

Fig. (1). Flowchart of systematic review process.

Table 1. Main characteristics of all the studies included in the systematic review.

Study	Country	Cell Culture	Substance Tested	Parameters Evaluated
Schmidt *et al*., 2010	Germany	SH-SY5Y, U-937	Amphetamine, Atomoxetine, Methylphenidate	A, C
Schmidt *et al*., 2010a	Germany	SH-SY5Y, U-937	Amphetamine, Atomoxetine, Methylphenidate	A, B
Schmidt *et al*., 2010b	Germany	SH-SY5Y	*Hypericum perforatum, Pinus radiata, Pinus pinaster*	A, B
Nam *et al*., 2014	Korea	SH-SY5Y	YY162*	A
Feio-Azevedo *et al*., 2017	Portugal	SH-SY5Y	Amphetamine e metabolites**	A, C
Bartl *et al*., 2010	Germany	PC12	Methylphenidate	A, B, C
Bartl *et al*., 2014	Switzerland	PC12	Polyunsaturated fatty acids	A, B
Craenenbroeck *et al*., 2006	Belgium	HEK293rtTA, L929sA, CHO	Pipamperone	A, C
Wakamatsu *et al*., 2009	Japan	HEK-293	Methylphenidate	A
Ludolph *et al*., 2010	Germany	TsA201	Atomoxetine	A
Knorle *et al*., 2012	Germany	JAR	*Sideritis scardica*	A, B
Suter *et al*., 2006	Switzerland	Human lymphocytes	Methylphenidate	A
Schwarz *et al*., 2014	Germany	Lymphoblastoid cell lines	Methylphenidate	A, C
Kittel-Schneider *et al*., 2016	Germany	Peripheral blood mononuclear cells	Methylphenidate	A
Zhao *et al*., 2007	China	CHO	*Fructus Psoraleae*	A
Salviano *et al*., 2015	Brazil	MDCK	Methylphenidate	A, B

Abbreviations: SH-SY5Y: neuroblastoma SH-SY5Y; U-937: Human monocytic U-927; PC12: Rat pheochromocytoma; HEK293rtTA cells: Human embryonic kidney cells expressing the tetracycline transactivator; HEK-293: Human embryonic kidney 293; TsA201: Transformed human embryonic kidney; JAR: Human choriocarcinoma; L929sA: pMx5-HT2AR cells mouse fibrosarcoma; CHO: Chinese hamster ovary; MDCK: Madin-darby canine kidney; *YY162: Mixture of "terpenoid-strengthened Ginkgo biloba" and "ginsenoside Rg3"; **4-hydroxyamphetamine e 4-hydroxynorephedrine; A: culture conditions; B: cell viability; C: gene expression.

cell lines, peripheral blood mononuclear cells, and cells isolated from animals (L929sA, CHO, MDCK) were reported. The evaluated therapeutic options included drugs (*e.g*. amphetamine, atomoxetine, methylphenidate, pipamperone), extracts of different plants and polyunsaturated fatty acids. All the studies presented data on the cell culture conditions, while 5 also evaluated cell viability, and 5 assessed gene expression. The results for the conduct and reporting of studies with the SYRCLE tool showed that all the studies described the conditions of cell culture at least. However, studies were imprecise or failed to present complete results for all of the tests. It is unclear whether studies were free of selective data reporting. Half of the studies were also 'unclear' about the acknowledgments or conflicts of interest related to the pharmaceutical industry.

3.1. Neuronal Cell Culture

From the 16 studies selected in our systematic search, five (31.3%) used the SH-SY5Y neuroblastoma [7, 8, 10, 33, 34] and two (12.5%) used the PC12 cell line [18, 32]. This first strain is a sub-line of the SK-N-SH cell, which was established in culture in 1970 from human metastatic neuroblastoma tissue [35]. One study acquired these cells from the European Collection of Cell Cultures (ECACC) - United Kingdom (UK), and other used the Korean Cell Line Bank - Seoul as a source of cells [10, 34]. The other studies did not report this information. The PC12 cell is a clonal cell line derived from a pheochromocytoma of the rat adrenal medulla [36]. Bartl *et al*. [18] acquired these cells through donation by researchers from the Technion Faculty of Medicine, Israel.

3.1.1. Culture Conditions for Neuronal Cells

In the seven included studies, the media used for cell growth was Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute medium (RPMI), with compositions that varied the concentration and condition of Fetal Bovine Serum (FBS) (varying from 3% heat-inactivated FBS to 15% FBS), the concentration of antibiotics (penicillin, streptomycin or gentamycin), and other supplements (*e.g*. amino acids). Other information, such as confluence, the use of trypsin to collect the cells, cell density and number of passages was reported only by Feio-Azevedo [10]. In all the studies, cells were maintained in a 5% CO_2 incubator. Four studies reported an incubation temperature of 37 °C [10, 18, 32, 34].

3.1.2. Phenotype and Cell Differentiation for Neuronal Cells

The SH-SY5Y cell line of neuroblasts can be differentiated into mature human neurons from methods based on the use of substances that trigger biochemical changes and may induce different neuronal phenotypes. One of the substances used is retinoic acid (RA), which is capable of inducing a cholinergic phenotype when applied in isolation or a dopaminergic phenotype when associated with phorbol ester and 2-O-tetradecanoylphorbol-13-acetate (TPA) [24, 37]. Only one study [10] reported the process of cell differentiation in the dopaminergic phenotype, using the method previously described by Ferreira *et al*. [38] in which the cells were exposed to RA and TPA in DMEM medium for 6 days. The PC12 cell line can differentiate to resemble sympathetic neurons when cultured in the presence of nerve growth factor (NGF) or several other compounds [36]. Bartl *et al*. [32] differentiated these cells with 50 ng/mL human NGF-b for 7 days according to the protocol used. The remaining studies did not report this information.

3.1.3. Cell Viability for Neuronal Cells

Three studies [8, 9, 11] quantitatively evaluated cellular viability the neuroblastoma SH-SY5Y using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide (MTT) assay, measuring lactate dehydrogenase (LDH) activity, or evaluating the adenosine triphosphate (ATP) content.

In the studies that performed the MTT test, neuronal cells were exposed to amphetamine, atomoxetine, methylphenidate or *Pinus radiata* D. Don, and *Pinus pinaster* Aiton extracts, and then incubated at 37 °C (overnight/24 h). Mitochondrial activity was evaluated at different times of exposure and substance concentrations. The final results revealed that none of the substances reduced neuronal cell survival compared with control cells [8, 9, 11]. The activity of LDH enzyme was measured by only one study [11] in which cell death was induced by concentrations of amphetamine (3.50 mM and 5.00 mM). The results showed no correlation between cell death and the exposed substance at the concentration and time analyzed (24 h and 48 h) [11, 37]. Two studies [8, 9] measured the cellular content of ATP, which was performed with a scintillation microplate counter. The cells were exposed to different substances (amphetamine, atomoxetine, methylphenidate and extracts of *Hypericum perforatum* L, *P. radiata* and *P. pinaster*) at different concentrations (500 and 5000 ng/mL) for 24 h at 37°C. There was a significant increase in ATP content only when exposed to the *H. perforatum* extract at a concentration of 5000 ng/mL.

The studies that evaluated cellular viability with the cells PC12 used XCELLigence and BrdU incorporation tests. Bartl *et al*. [18] non-invasively evaluated cell viability using an xCELLigence Real-Time Cell Analyzer. In this case, the culture medium of PC12 cells was changed to DMEM containing 4.5 mg/mL glucose, supplemented with 1% FBS and 150 μM fatty acid-free bovine serum albumin (BSA). The results demonstrated that the combination of polyunsaturated fatty acids significantly increased cell viability. The assay of 5-bromo-2-deoxyuridine (bromodeoxyuridine or BrdU) performed by Bartl *et al*. [32] showed that concentrations of 1, 10, and 100 nM of methylphenidate activated DNA synthesis in PC12 cells compared to the controls, whereas higher concentrations of the drug (1-100 μM) did not influence cell proliferation.

3.1.4. Gene Expression for Neuronal Cells

Among the existing methods to evaluate gene expression, reverse transcription polymerase chain reaction (RT-PCR) is a widely used tool to amplify and detect mRNA [39]. This was the technique used by Schmidt *et al*. [33] to verify the effect of amphetamine, atomoxetine and methylphenidate in different concentrations (50, 500 and 5000 ng/mL) on the expression of the 8-hydroxyguanine glycosylase 1 (hOGG1). The results revealed that the evaluated drugs decreased the expression of this enzyme, which is directly related to the level of oxidative products of the DNA, suggesting a neuronal protective effect of psychostimulants and atomoxetine. One study [32] evaluated the effect of methylphenidate on gene expression in PC12 cells. Total RNA was extracted by the RNeasy kit and quality was evaluated on an agarose gel.

3.2. Non-neuronal Cell Cultures

Among the 16 studies, nine (56.25%) used non-neuronal cultures or cells isolated from animals . These cell lines were accessed through Cytomyx Ltd. (Cambridge, UK), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (Braunschweig, Germany) [9], and from the Biochemistry Institute of São Paulo University, Brazil [21].

3.2.1. Culture Conditions for Non-neuronal Cells

All of the studies described culture conditions. The media used for cell growth were Minimum Essential Medium (MEM) in 3 of the studies, DMEM in one study or RPMI-1640 in 5 studies; their compositions varied in relation to supplementation of the medium, represented by the addition of FBS (varying from 10 to 20%), different antibiotics and other supplements (*e.g*. amino acids). Incubation was performed at 37 ºC and 5% CO_2.

3.2.2. Main Methodologies for Non-neuronal Cells

The studies were methodologically designed according to the type of cells and their objectives. Most of the studies were carried out to verify the cytotoxic potential of test substances tested or their influence on gene expression, electrophysiological test, levels of transporters and others tests (Table **2**). Cell viability and gene expression were evaluated by two (22.2%) and two (22.2%) studies, respectively.

3.2.3. Cell Viability for Non-neuronal Cells

Two studies performed viability and cell proliferation tests using LDH and MTT.

Knorle *et al*. [9] measured the LDH activity from the exposure of JAR cells to extracts of *S. scardica* at concentrations of 50 and 500 μg/mL. The cells were incubated at 20 ºC (3 h). There was no significant difference between the cells treated with the extracts and control cells. The studies performing the MTT assay [21] also observed no reduction in cell viability when MDCK cells were treated with methylphenidate at concentrations of 80 to 1.25 μL/mL.

Table 2. Main tests reported in the studies with non-neuronal cells cultures.

Authors, Year	Cell Culture	Study Objectives	Cell Viability	Gene Expression	Others
Craenenbroeck *et al*., 2006	HEK293rtTA	To evaluate the effect of pipamperone on D4 receptor expression	-	qPCR	Radioligant Western blot
Wakamatsu *et al*., 2009	HEK 293	To evalute the effect of methylphenidate on the cardiovascular system	-	-	Electro-physiological
Ludolph *et al*., 2010	TsA201	To evaluate the effect of atomoxetine on glutamate receptors	-	-	Electro-physiological
Knorle *et al*., 2012	JAR	To evaluate the effect of *Sideritis scardica* on monoamine transporters	LDH	-	Effect on transporters
Suter *et al*., 2006	Human lymphocytes	To evaluate the clastogenic effect of methylphenidate	-	-	Chromosome aberration
Schwarz *et al*., 2014	Lymphoblastoid Cell Lines	To evaluate the effect of methylphenidate on gene expression regulation	-	qRT-PCR Microarray	-
Kittel-Schneider *et al*., 2016	Peripheral blood mononuclear cells	To evaluate the cytogenetic effects of long-term treatment with methylphenidate	-	-	Micronucleus
Craenenbroeck *et al*., 2006	L929sA	To evaluate the effect of pipamperone on D4 receptor expression		qPCR	Radioligant Western blot
Craenenbroeck *et al*., 2006	CHO	To evaluate the effect of pipamperone on D4 receptor expression	-	qPCR	Radioligant Western blot
Zhao *et al*., 2007	CHO	To evaluate the effect of *Fructus Psoraleae* on noradrenaline and dopamine transporters	-	-	Effect on transporters
Salviano *et al*., 2015	MDCK	Evaluate the effect of methylphenidate on the renal system	MTT	-	-

Abbreviations: HEK293rtTA cells: Human embryonic kidney cells expressing the tetracycline transactivator HEK-293: Human Embryonic Kidney; TsA201: Transformed human embryonic kidney; JAR: Human choriocarcinoma; L929sA: Cells mouse fibrosarcoma; CHO: Chinese Hamster Ovary; MDCK: Madin-Darby Canine Kidney; Receptor D4: Receptor de dopamine 4; 6-OHDA: 6-hydroxydopamine; qRT-PCR: quantitative Real Time PCR; *Eicosapentaenoic acid e docosahexanoic acid e gamma-linolenic acid.

3.2.4. Gene Expression for Non-neuronal Cells

Two studies [13, 19] evaluated the effect of methylphenidate on gene expression in LCL and CHO cells. Total RNA was extracted by the RNeasy kit and evaluated for quality on an agarose gel. In addition, Schwarz *et al*. [19] performed microarray hybridization.

The results showed that the effects of long-term methylphenidate treatment were observed on the expression of the ATXN1, HEY1, MAP3K8 and GLUT3 genes, while the effects of acute treatment were observed on the expression of NAV2 and ATXN1 in patients with ADHD, confirmed by quantitative Real-Time PCR (qRT PCR) analysis [19]. Craenenbroeck *et al*. [13] used qPCR to verify that the drug pipamperone does not increase the expression of dopamine 4 receptor mRNA in the CHO FLAGDRD4 cell line.

3.2.5. Other Tests for Non-neuronal Cells

The electrophysiological tests evaluated the effect of methylphenidate and atomoxetine on cells [16, 17]. The first drug was tested to verify its effect on the cardiovascular system from the delayed rectification potassium current (IKr) analysis in human ether-a-go-related (hERG) human HEK 293 cells. Methylphenidate was applied to the cell culture at different concentrations (0.1, 0.3 and 1 μg/mL) but none of them inhibited IKr, which suggests that this drug does not alter the ventricular repolarization process (prolongation of QT interval) at the recommended therapeutic dosage levels [16].

The effects of atomoxetine on glutaminergic receptors on TsA201 cells transfected with cDNAs, which encode N-methyl-D-aspartate (NMDA) receptors, were also evaluated. Atomoxetine exerted a dose-dependent antagonistic effect on NMDA receptors at lower concentrations, which suggests a relationship between glutaminergic transmission and the development of ADHD [17].

The extracts of the plant species *Sideritis scardica* Griseb. and *Fructus Psoraleae* inhibited the monoamine transporters in tests performed with human (JAR) and animal (CHO) cells, respectively [9, 15]. According to the authors, JAR cells were chosen due to the expression of human serotonin transporter (hSERT), while CHO cells were chosen due to the expression of rat dopamine transporter (rDAT), rat serotonin transport (rSERT), mice γ-aminobutyric acid or GABA transporter (mGAT-1) and human noradrenaline transporter (hNET).

Other tests were also performed. Craenenbroeck *et al*. [13] used radioligand, western blot and tests in HEK293rtTA, L929sA and CHO cells to evaluate that pimperone acts as a pharmacological chaperone and increases the expression level of the dopamine D4 receptor. This receptor may be involved with the development the ADHD. Chromosomal aberration and micronucleus tests on isolated human lymphocytes were performed to evaluate possible cytotoxic effects of the drug methylphenidate. The results demonstrated the absence of a clastogenic effect in these cells after treatment with D, L-methylphenidate in concentration up to 10 mM [14]. Long-term treatment did not lead to cytogenetic effects [20].

4. DISCUSSION

This is the first systematic review to synthesize information on cell cultures as research models for assessing therapeutic options in ADHD. In addition to contributing further evidence on the safety and toxicity profile of well-established drugs (methylphenidate, amphetamine and atomoxetine), it was possible to verify the use of these models for the investigation of potential therapeutic substances, such as plant extracts and polyunsaturated acids.

The choice of pharmacological treatments for ADHD is mostly based on physiopathological aspects of the disease, such as alterations in the dopaminergic, noradrenergic and serotonergic neurotransmission [40]. Currently, psychostimulants (*e.g*. amphetamine and methylphenidate) and noradrenaline reuptake inhibitors (*e.g*. atomoxetine) are considered first and second line treatments, respectively [11]. Although the therapeutic effect (efficacy and safety) of these drugs has been proven in *in vivo* studies and clinical trials [11], the *in vitro* studies included in our review also show that these drugs did not interfere with cell viability or gene expression, nor present significant toxicity. Moreover, cell culture assays suggest that psychostimulants and atomoxetine can protect DNA against oxidative stress, because they decrease the expression of the hOGG1 enzyme in cell lines, which has a positive correlation with the level of products of oxidative lesions to the DNA [33]. This is important to guide further investigation into the neuropathophysiology of ADHD, which has still not been fully elucidated.

Our review also found other substances that act at the level of monoamine transporters, making them possible therapeutic options for neurological disorders. Among the extracts of plants and polyunsaturated fatty acids, two substances presented promising results: the extracts *S. scardica* and *Fructus Psoraleae*. The results showed that these substances did not alter cell viability, but inhibited dopamine, norepinephrine and serotonin transporters, which are usually altered in patients with ADHD. However, studies to better characterize and isolate the potentially active compounds of the extracts are needed [9, 15]. Moreover, additional tests such as *in vitro* electrophysiological studies should be performed to investigate cellular mechanisms of action, together with *in vivo* tests for safety assessment [41].

To obtain reliable results, *in vitro* studies with cell cultures should be appropriately conducted and reported. Sev-

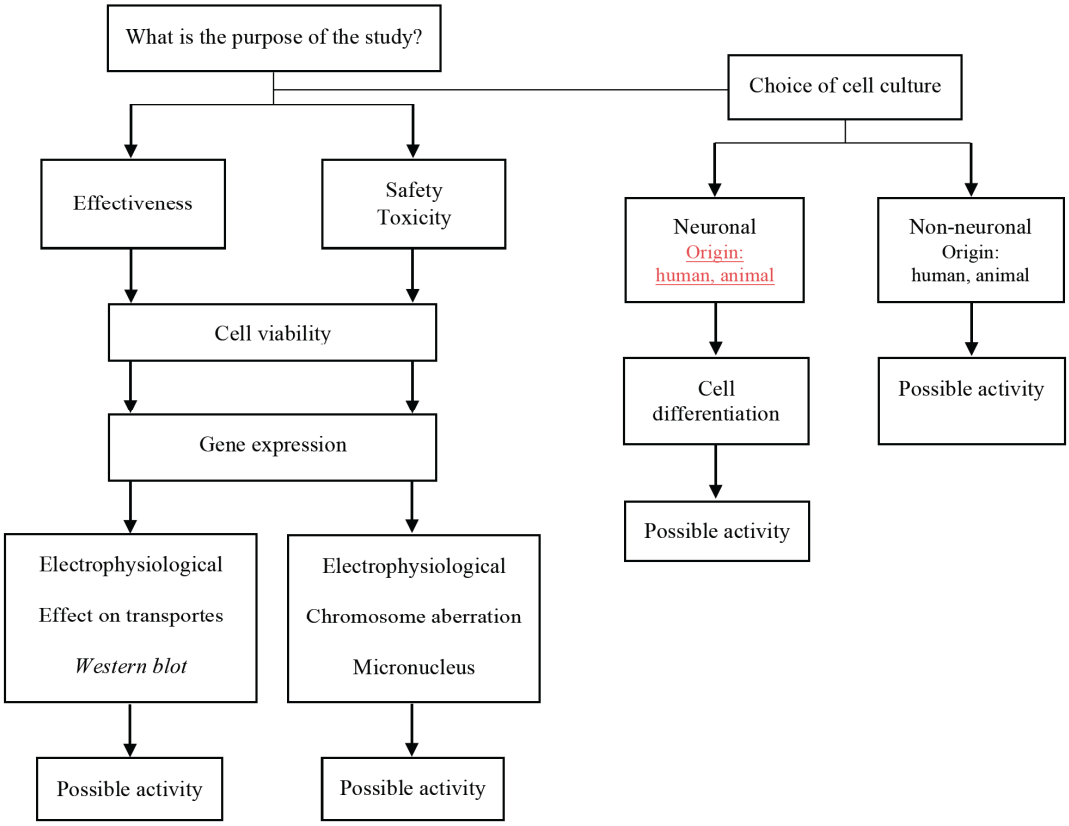

Fig. (2). Flowchart with the main methodological steps for *in vitro* research investigating therapeutic options for ADHD.

eral studies have documented concerns about the lack of reproducibility in scientific studies, particularly preclinical studies involving cells and animals [42-44]. Quality standards and good practices are generally not well defined for *in vitro* methods and *in vivo* models, and have not been integrated into preclinical research laboratories [45]. Several factors are commonly attributed to reduce methodological quality, including the poor design of experiments, the lack of training of investigators, and insufficient reporting of results or the withholding of technical details. As a consequence, the literature becomes irreproducible and unreliable, the bench-to-bedside time for new drugs is negatively affected, and the resources needed for clinical development significantly increase [45, 46].

Considering how the *in vitro* studies included in our review were conducted, and the most appropriate methodological aspects to perform cell cultures for ADHD, we propose the research process depicted in Fig. (**2**). This flowchart presents the main steps that should be considered by researchers when performing a study aiming to evaluate the effects and toxicity of substances exposed to neuronal and

Table 3. Checklist for conduct and reporting experimental *in vitro* studies.

Checklist Item	Reported on Page Nº
CELL CULTURE	
Identification of culture type	
Identification of cell type	
Origin of cells (human, animal)	
In case of cells isolated from tissues, report isolation technique and variables involved	
Source of access of cells (collection of cell biology, donation by laboratories)	
In case of collection of cell biology, report the product/catalog number	
Growth medium used	
Growth medium supplementation components (serum, antibiotics, micronutrients, others). Report name, concentration, percentage used and brand	
Frequency of change of growth medium	
pH of the growth medium	
Confluence	
Use of trypsin or other substance to collect the cells. Report name, concentration, percentage	
Cellular density	
Number of passages	
Incubation temperature (exact 0.0 °C)	
Atmosphere conditions (exact 0.0% CO_2)	
METHODS	
Technique name	
Report whether the technique has been developed and validated or reproduced	
In case of reproduced technique, report if there were adaptations and what were they	
In case of use of substance that promotes cellular alteration, report name, function, concentration, percentage, exposure time	
Time period of each step	
Temperature (exact 0.0 °C)	
Atmosphere conditions (exact 0.0% CO_2)	
Equipment used	
Type of statistical analysis and level of significance	
Software for statistical analysis	

non-neuronal cells, including the selection of the cell culture and test to be performed based on the study's goals.

The selection and maintenance of cells is an important first step. Often, cells used in the cultures are acquired by collections of cell biology or donated by research laboratories. Laboratory conditions, including the choice of medium and the type and serum concentrations, should be optimized for each type of cell, since these factors promote cell survival and proliferation, and modulate the biological behavior of cells, influencing the ability of differentiation and metabolic profile [1, 47, 48]. The composition of the growth medium is established differently according to the sources of access [27]. Bovine, adult, newborn or fetal (FBS) serum are the most commonly used [49]. Other substances may be added for the maintenance of culture media. Antibiotics (penicillin, streptomycin, gentamicin, and tylosin) are added to prevent bacterial and fungal contaminants [50], while non-essential amino acid supplementation, as observed in DMEM and RPMI-1640, is used to promote growth and increase cell viability [22]. Moreover, the addition of sodium pyruvate may exhibit a cytoprotective activity against oxidative stress, preventing cell death induced by hydrogen peroxide [51, 52].

As sources of energy for cellular media, glucose is often added in the form of carbohydrates (primary source), and the essential amino acid glutamine (secondary energy source for cellular metabolism). Attention should be paid, however, to the maximum permissible concentrations of these components to maintain normal cell activities [22, 53].

For SH-SY5Y neuroblastoma cells, the process of differentiation into mature human neurons involves the addition of neurotrophic factors, factors secreted by human stem cells (hNSC), extracellular matrix proteins, and the gradual withdrawal of serum, as well as the use of retinoic acid [37]. This combination results in rapid and effective differentiation, with a higher percentage of neurons obtained [54]. The advantage of this methodology is the possibility of obtaining more robust results for comparison with *in vivo* models [37]. Both neuronal cells and non-neuronal cells can also be tested for cell activity. The substances to be tested, as well as their concentration and time of exposure should also be defined in the study's protocol. Another critical step in *in vitro* research is the determination and optimization of methods for cell viability. This enables one or more cellular functions such as mitochondrial activity (MTT), membrane integrity and cellular metabolism (LDH), DNA replication (BrdU incorporation), and ATP production, among others, to be evaluated [25], which may guide the elucidation of molecular pathways. Additionally, gene expression tests may broadly show cellular reactions due to a pathological condition or the effect of pharmacological treatments [23, 54-56]. Since many factors may affect these assays, standardized conditions are needed to obtain accurate results [55-57].

Besides optimized practices for planning and conducting *in vitro* studies, the reporting of results should also be standardized. In the healthcare area, the concept 'core outcome sets' represents a standardized collection of outcomes that should be measured and reported, which facilitate interpretation and generate consistent outcomes across studies [58, 59]. Moreover, well-established checklists and recommendations with a minimum set of items for reporting are required for the publication of clinical and observational studies [28, 29]. For preclinical studies, only a few recommendations such as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist exist to guide study planning, execution, data analysis and reporting [56]. Thus, we suggest a minimum checklist (Table **3**) that should be completed by researchers using cell cultures prior to publication. This checklist was built based on the information reported in the studies included in our review and complemented with published literature on cell-lineage and cell culture methods (Guidance on Good Cell Culture Practice - GCCP) [60]. Together with the flowchart of the research process, the checklist aims to improve laboratory protocols, guide further standardized *in vitro* and *in vivo* research, facilitate the interpretation of data by the readers and allow result reproducibility.

Our research has some limitations. Few studies were included in the review, which may limit the generalization of the results to other fields. However, no further studies were added by manual searches. Given the nature of the data and the moderate methodological quality of the studies, other quantitative analyses were not possible.

CONCLUSION

This is the first systematic review to describe the methodological aspects for planning, performing and reporting *in vitro* studies with cell cultures in ADHD. To date, the SH-SY5Y neuroblastoma cell line is the most widely used for the evaluation of cell proliferation and viability and gene expression. However, non-neuronal cells were shown to be useful for evaluation of the safety and toxicity of drugs and extracts of plants. Given the heterogeneity in the conduct of preclinical studies, we proposed a flowchart of the main methodological steps that should be followed by researchers during the development of laboratory protocols and further *in vitro* investigation. Additionally, we created a checklist with minimum reporting items that should be used by authors prior to publication.

CONSENT FOR PUBLICATION

Not applicable.

STANDARD OF REPORTING

The study has been financially supported by PRISMA guidelines have been followed in this study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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