Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

ESCOLA NACIONAL DE SAÚDE PÚBLICA
SERGIO AROUCA
ENSP

"Métodos alternativos ao uso de animais na toxicologia de agrotóxicos: uma revisão sistemática e uma proposta de método para avaliação de anticolinesterásicos"

por

Róber Freitas Bachinski

Dissertação apresentada com vistas à obtenção do título de Mestre em Ciências na área de Saúde Pública e Meio Ambiente.

Orientador: Prof. Dr. Armando Meyer

Rio de Janeiro, agosto de 2011.

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

ESCOLA NACIONAL DE SAÚDE PÚBLICA
SERGIO AROUCA
ENSP

Esta dissertação, intitulada

"Métodos alternativos ao uso de animais na toxicologia de agrotóxicos: uma revisão sistemática e uma proposta de método para avaliação de anticolinesterásicos"

apresentada por

Róber Freitas Bachinski

foi avaliada pela Banca Examinadora composta pelos seguintes membros:

Prof. Dr. Moacelio Veranio Silva Filho

Prof.ª Dr.ª Paula de Novaes Sarcinelli

Prof. Dr. Armando Meyer – Orientador

Dissertação defendida e aprovada em 01 de agosto de 2011.

Catalogação na fonte
Instituto de Comunicação e Informação Científica e Tecnológica
Biblioteca de Saúde Pública

B123 Bachinski, Róber Freitas
Métodos alternativos ao uso de animais na toxicologia de agrotóxicos: uma revisão sistemática e uma proposta de método para avaliação de anticolinesterásicos. / Róber Freitas Bachinski. -- 2011.
68 f. : tab. ; graf.

Orientador: Meyer, Armando
Dissertação (Mestrado) – Escola Nacional de Saúde Pública Sergio Arouca, Rio de Janeiro, 2011

1. Alternativas ao Uso de Animais-métodos. 2. Praguicidas. 3. Toxicologia. 4. Acetilcolinesterase. 5. Revisão. I. Título.

CDD - 22.ed. – 632.95

DEDICATÓRIA

Aos meus pais que sempre prezaram por minha educação, confiaram nas minhas decisões e nunca mediram esforços para me ajudar a construir o meu caminho.

AGRADECIMENTOS

Muitas pessoas são responsáveis pelo que sou e pelo que penso. Como um ser social, sou formado pelas ideias que chegam a mim e muitas não posso saber sequer de onde surgiram. Teria que agradecer a todos os pensadores que já li; a todos aqueles a quem escutei, mesmo que não concorde com eles, pois isso me ajudou a refletir e a formar novas opiniões. Porém isso seria sem sentido, pois muitos deles já morreram há séculos. Outros nem sequer pensam que são importantes ou que atingiram quilômetros de distâncias. Espero que minha forma de agradecimento seja retribuindo essas contribuições através do meu trabalho, inspirando novas ideias.

Aqui me limito apenas aos agradecimentos especiais e às pessoas inesquecíveis:

Aos meus pais, Rosemary e Roberto,já citados na dedicatória.

Aos meus amigos Naza e João, um grande achado na minha vida, por sempre me apoiarem e me aconselharem.

Aos meus colegas Lara, Poli, Enrico, Bia e Patrícia por me agüentarem no início do curso, quando pensava que nada iria melhorar.

À equipe do Departamento de Farmacologia e Toxicologia do INCQS, em especial ao Octavio Presgrave, Cristiane Caldeira, Eloisa Alves, que me apresentaram à prática das pesquisas em métodos alternativos ao uso de animais em toxicologia.

À equipe da Biblioteca da ENSP, em especial a Gizele Rocha, que me ensinou os primeiros passos em revisões bibliográficas e me apoiou muito no mestrado, quando tive muitas dificuldades.

À equipe da Unidade de Pesquisa Clínica da UFF, onde tive um apoio extraordinário para o desenvolvimento da minha dissertação, desde materiais, infraestrutura, apoio técnico, amizade... em especial à Adriana, que me apoiou na cultura de células; à Renata que me auxiliou na primeira reunião da UPC, incentivando a minha entrada; à Ângela, sempre de bom humor para nos receber; ao Guto por me aceitar na unidade e como aluno de doutorado, a todos os outros colegas que compartilham a unidade e fazem de lá um ótimo local de trabalho.

Ao professor Moacelio por ter me ensinado muito sobre acetilcolinesterase, métodos de análises, e por pensar comigo alguns experimentos. Também me apresentou as ideias sobre o sistema de ensino baseado em problemas (PBL).

Devemos atribuir o mesmo peso para interesses semelhantes para todos aqueles que são atingidos por nossos atos, ou seja, um interesse é um interesse, seja lá de quem for, o que nos leva a uma condenação radical do racismo, do sexismo e do especismo.

Peter Singer, na obra “Ética Prática”, publicada pela primeira vez em 1979.

Entretanto, haverá algum motivo para se tolerar que atormentemos os animais? Sim. Vários (...), houve um tempo – lamento dizer que em muitos lugares ele ainda não passou – no qual a maior parte da nossa espécie, sob a denominação de escravos, foram tratados pela lei exatamente no mesmo pé que, por exemplo, na Inglaterra, as raças animais inferiores ainda são tratadas hoje. Pode vir o dia em que o restante da criação animal adquira aqueles direitos que nunca lhes deveriam ter sido tirados, se não fosse por tirania. Os franceses já descobriram que a cor preta da pele não constitui motivo algum pelo qual um ser humano pode ser entregue, sem recuperação, ao capricho do verdugo. Pode chegar o dia em que se reconhecerá que o número de pernas, a pele peluda, ou a extremidade do *os sacrum* constituem razões igualmente insuficientes para abandonar um ser sensível à mesma sorte. Que outro fator poderia demarcar a linha divisória que distingue os homens dos outros animais? Seria a faculdade de raciocinar, ou talvez a de falar? Todavia, um cavalo ou um cão adulto é incomparavelmente mais racional e mais social e educado que um bebê de um dia, ou de uma semana, ou de um mês. Entretanto, suponhamos que o caso fosse outro: mesmo nesta hipótese, que se demonstraria com isso? O problema não consiste em saber se os animais podem raciocinar; tampouco interessa se falam ou não; o verdadeiro problema é este: podem eles *sofrer*?

Jeremy Bentham, na obra “Uma introdução aos Princípios da moral e da Legislação”, publicada pela primeira vez em 1789

RESUMO

O uso de agrotóxicos utilizados no controle de organismos indesejados à saúde humana ou a produção agrícola tem aumentando continuamente. De encontro com essa tendência, há diversos estudos que apontam que o uso desses produtos possuem um impacto danoso sobre a saúde ambiental e humana. A fim de alcançar uma regulamentação adequada sobre a utilização de pesticidas e a exposição a esses produtos, são solicitados vários testes toxicológicos, feitos ainda com modelos animais, *in vivo*. No entanto, a extrapolação dos resultados obtidos a partir de outras espécies animais para os seres humanos e para outras espécies não-alvos é um assunto de intenso debate. Nesse meio tempo, uma grande quantidade de animais é usada em testes toxicológicos em todo o mundo, reforçando o imperativo ético que responsibiliza a comunidade científica a desenvolver métodos alternativos finais para estes testes. Neste contexto, o objetivo deste estudo foi revisar sistematicamente a literatura científica sobre métodos alternativos para ensaios de toxicidade de pesticidas. Esta revisão sistemática mostrou que há poucos estudos de métodos alternativos ao uso de animais em testes toxicológicos de pesticidas e eles estão concentrados em apenas dois campos. Outras áreas da toxicologia podem ser exploradas por novos métodos. As pesquisa sobre métodos substitutivos devem ser incentivadas, para que possa reduzir ao máximo e abolir o uso de animais na pesquisa.

Palavras-chaves: Alternativas ao uso de animais, praguicidas; toxicologia, acetilcolinesterase.

ABSTRACT

The use of pesticides to control living organisms that threaten human health and crops/food supply is continuously increasing. Conflicting with that, there are several evidences that such increase may pose a danger to environmental and human health. In order to achieve proper regulation of pesticide use and exposure, several toxicological tests are available employing animal testing. However, the extrapolation of the results obtained from such species to humans and other non-target species is still a matter of intense debate. In the meantime, a large amount of animals is used in toxicological tests worldwide, reinforcing the ethical imperative to develop final alternative methods to these tests. In this context, the aim of this study was to systematically review the scientific literature about alternative methods to pesticide toxicity assays. Therefore, this systematic review showed that only few studies issue alternative methods to the use of animals in pesticide toxicological tests and they are concentrated in only two fields. Other fields can be explored by new methods. Research on replacement methods needs to be encouraged as it could lead to significantly reduce the current massive use of animals in experimental science.

Key-words: Animal Use Alternatives, pesticides, toxicology, acetylcholinesterase

LISTA DE ABREVIATURAS E SIGLAS

Ach - Acetilcolina

AchE - Acetilcolinesterase

AD – Alzheimer Disease (Doença de Alzheimer)

AT – Agente tóxico

ATC - Iodeto de acetilcolina

ATCC – Coleção de Culturas Americana (*American Type Culture Collection*)

ATP – Adenosina-trifosfato

ATPase - Adenosina-trifosfatase

B50 – Linhagem celular de neuroblastoma de rato

CTTAEA – Comite sobre testes de toxicidade e avaliaçaõ de agents ambientais – Conselho de Pesquisa Nacional (Committee on Toxicity Testing and Assessment of Environmental Agents - National Research Council)

EC50 - Effective concentrations 50%

ENSP – Escola Nacional de Saúde Pública Sérgio Arouca

EU – União Européia

HTD - Highest tolerated dose

IC50 - Growth Inhibitory Concentration of 50%

KB - Kenacid blue

LC50 - Lethal concentration 50%

μg - microgramas

mg - miligrama

NB2a, N2a - neuroblastomas de camundongos

NRU - Neutral red uptake

NT2 - Carninoma embrionário humano

PC12 - Feocromocitoma de rato

REACH - Registration, Evaluation and Authorisation of Chemicals

SK-N-SH, SH-SY, SH-SY5, SH-SY5Y, SH-IN, SH-EP, SH-FE, SK-N-BE, SK-N-MC, MC-IIE, MX-IXC – Linhagens e derivações de neuroblastomas humanos.

SNC – Sistema Nervoso Central

UDP - Up-and-down procedure

SUMÁRIO

1 – INTRODUÇÃO 13
2 – REFERENCIAL TEÓRICO 15
2.1. AGROTÓXICOS 15
2.4. LINHAGEM CELULAR DE NEUROBLASTOMA HUMANO (SH-SY5Y) 25
3 – OBJETIVOS 27
3.1. GERAL 27
3.2. ESPECÍFICOS 27
4 – MATERIAIS E MÉTODOS 28
5 – RESULTADOS 29
5.1. ARTIGO 1: What Do We Know About Alternative Methods to Pesticide Toxicology? A Systematic Review. 29
Abstract 30
Introduction 30
Methods 31
Searching 31
Selection 32
Data abstraction 32
Quantitative data synthesis 32
Results 33
Acute toxicity studies 33
Ecotoxicity 34
Hepatotoxicity 36
Eye irritation 37
Eye irritation - Quantitative data synthesis 38
Skin permeability 39
Neurotoxicity 39
Reproductive toxicity 40
Adrenotoxicity - endocrine disruptors 40
Discussion 40

Limitations 42
Acknowledgments 42
References 43
Annexes 46
5.2. ARTIGO 2: A high-throughput test using cellular acetylcholinesterase activity without cell injury 51
Abstract 52
Introduction 53
Materials and Methods 54
Cell culture 54
Determination of acetylthiocholine hydrolysis in possible test media 54
Determination of acetylcholinesterase activity 55
Acetylcholinesterase inhibition test 55
XTT Assay 55
Results and Discussion 55
Photometric analysis of TNB (thionitrobenzoate) 56
Selection of test medium 56
Acetylcholinesterase detection 57
Acetylcholinesterase inhibition and cellular viability 58
References 61
6 – CONSIDERAÇÕES FINAIS 63
7 – REFERÊNCIAS 64

1 – INTRODUÇÃO

O objetivo central da toxicologia é estudar os efeitos adversos de substâncias para prevenir danos à saúde humana. Com o avanço da toxicologia, podem-se criar melhores políticas públicas, normatizações de usos de materiais e recomendações de cuidados ao manipular ou expor seres humanos a certas substâncias. Porém, as complexidades das relações do homem com ele mesmo e com o ambiente fazem com que os métodos de análise sejam rapidamente defasados, frente a novos desafios.

Além disso, devemos pensar que a ciência não está à parte da sociedade, mas sim que ela é uma ferramenta da sociedade. A ciência não possui suas próprias normas e seus próprios imperativos éticos, mas deve seguir as normas sociais e os imperativos éticos universais, como limites. Não basta a uma metodologia que ela seja eficiente naquele teste a que se propõe, mas sim que ela seja socialmente aceita, eticamente defensável, economicamente viável e rápida para poder identificar riscos à saúde humana e ambiental.

Nesta linha de pensamento, primeiramente foi feita uma análise das publicações científicas em diversas bases de dados, identificando-se as pesquisas publicadas com o interesse em contribuir para o avanço dos métodos alternativos ao uso de animais em relação aos estudos de toxicidade dos agrotóxicos. A partir dessa análise, pode-se descrever quais avanços já houve no desenvolvimento de métodos alternativos, mas também por quais caminhos seguir. Ainda pode-se utilizar essas metodologias como guias para novas investigações para todas as outras substâncias de interesse toxicológico.

Entretanto falta muito para que se consiga um avanço suficiente para a substituição do uso de animais na ciência. Os incentivos às pesquisas em métodos alternativos são baixos, o interesse é incipiente e o assunto, pouco discutido na academia. Buscando contribuir à toxicologia com o

desenvolvimento de alternativas que se escolheu a análise de neurotoxicidade baseada na inibição da Acetilcolinesterase, um mecanismo de ação bastante explorado para armas químicas na Segunda Grande Guerra e herdado pela pesquisa em agroquímicos para combate das espécies animais indesejadas ao modelo superlativo de produção agrícola.

2 – REFERENCIAL TEÓRICO

2.1. AGROTÓXICOS

2.1.1. Importância e impacto do uso de agrotóxicos

Legalmente, os agrotóxicos são definidos pela lei 7.802/98, sendo qualquer produto ou agentes (físicos, químicos ou biológicos) utilizados nos setores de produção, armazenamento ou beneficiamento de produtos agrícolas ou na proteção de florestas e ecossistemas (urbano ou rural), destinado a modificar a composição da flora ou fauna a fim de preservá-las da ação danosa de outros seres vivos, ou ainda produtos empregados como desfolhantes, dessecantes e estimulantes e inibidores de crescimento (BRASIL, 1989). Essa definição trás o caráter positivo dos agrotóxicos. Através deles é possível o controle de animais vetores de doenças de grande impacto na saúde da população, no controle de animais que colocam em risco a produção de alimentos em fazendas, ou na armazenagem pós-colheita ou das sementes antes do plantio/brotamento. Além disso, são utilizados no combate a animais parasitas de outras criações de animais, no benefício ao conforto do lar, no combate de insetos indesejados. Basicamente, todos os ambientes urbanos possuem ligação direta com agrotóxicos com algum desses objetivos.

A facilidade e a eficiência dos agrotóxicos no combate a esses animais indesejados, assim como o fácil acesso, fez com que a exposição humana a esses produtos, a doses que colocam em risco aguda ou cronicamente a saúde da população, chegasse a números preocupantes. Pelos dados acumulados em tabelas publicadas pelo SINETOX, do ano de 2005 a 2009, 786 pessoas obtiveram sucesso no suicídio utilizando agrotóxicos de uso agrícola no Brasil (tabela 1), resultando no suicídio como principal morte por agrotóxicos, 84,7% dos óbitos registrados de 2005 a 2009 no Brasil.

Tabela 1: Óbitos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, segundo principais circunstâncias, acumulado de 2005 - 2009.

REGIÃO	OCUPACIONAL (%)	SUICÍDIO (%)	TOTAL (%)
Norte	0 (0,0)	11 (1,2)	16 (1,7)
Nordeste	2 (0,2)	382 (41,2)	426 (45,9)
Sudeste	2 (0,2)	135 (14,5)	164 (17,7)
Sul	7 (0,8)	146 (15,7)	181 (19,5)
Centro-Oeste	6 (0,6)	112 (12,1)	141 (15,2)
Total	17 (1,8)	786 (84,7)	928 (100,0)

Compilado de SINITOX 2009b, 2009d, 2009f, 2010b, 2011b

Pela mesma fonte, podemos notar que mais de 12 mil pessoas, nesse mesmo período, foram cadastradas por apenas se intoxicarem voluntariamente, objetivando o mesmo fim. Ainda, mais de outras 7 mil pessoas se intoxicaram não voluntariamente, durante a exposição aos agrotóxicos de uso agrícola, durante o trabalho (tabela 2). Segundo estimativas da Organização Pan-Americana de Saúde (OPAS), para cada caso registrado de intoxicação por agrotóxicos outros 50 casos de intoxicação ocorreram sem notificação ou com notificações errôneas (SOBREIRA & ADISSI, 2003). O fácil acesso aos agroquímicos facilita o uso deles, pelo menos e não somente, como arma de auto-extermínio.

Tabela 2: Casos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, segundo principais circunstâncias, acumulado de 2005 - 2009.

REGIÃO	AMBIENTAL (%)	OCUPACIONAL (%)	TENTATIVA DE SUICÍDIO (%)	TOTAL (%)
Norte	0 (0,0)	32 (0,1)	197 (0,7)	459 (1,6)
Nordeste	12 (0,0)	274 (1,0)	4.335 (15,3)	5.897 (20,8)
Sudeste	58 (0,2)	3.860 (13,6)	4.246 (15,0)	11.410 (40,3)
Sul	15 (0,1)	2.588 (9,1)	2.711 (9,6)	7.592 (26,8)
Centro-Oeste	27 (0,1)	671 (2,4)	1.356 (4,8)	2.944 (10,4)
Total	112 (0,4)	7.425 (26,2)	12.845 (45,4)	28.302 (100,0)

Compilado de SINITOX 2009a, 2009c, 2009e, 2010a, 2011a

O mercado de agrotóxicos, no Brasil, movimentou em 2008 um montante de 7 bilhões de reais, colocando-o como responsável pelo consumo de 86% desses produtos em toda a América Latina (ANVISA, 2009). Perez e colaboradores (2007) alertam que os países em desenvolvimento

representam 30% do mercado global desses produtos. E ainda, segundo dados apresentados por esses mesmos autores, os menores índices de exposições a agrotóxicos através do trabalho ou por contaminações ambientais seriam de 3% das populações estudadas, podendo chegar a 23%. Ainda, alertam sobre o aumento do risco ocasionado pelos agrotóxicos, quando atingem populações mais vulneráveis, como com baixo nível de escolaridade, falta ou de uma política informativa sobre os perigos dos agrotóxicos fraca e ineficiente, pouca atenção ao descarte de materiais utilizados no transporte, embalagem e aplicação dos produtos e ao manejo e falta de controle sobre comercialização e acesso aos agrotóxicos.

O ônus devido o uso, excesso e a falta de zelo com esses produtos, tanto no ambiente urbano quanto rural, não são pagos apenas com o furto das vidas, voluntária ou involuntariamente, mas no tratamento de doenças e de intoxicações, sanadas pelo ou responsabilizadas ao Estado.

2.1.2. Classes e mecanismo de ação dos agrotóxicos

A domesticação dos animais e das plantas foram as bases para a formação das aldeias, da transição dos grupos errantes aqueles fixos. A possibilidade da produção do alimento fez com que o homem pudesse se associar à terra, abrindo um novo mundo de possibilidades, de relações entre os homens e entre os homens e os compartimentos bióticos e abióticos do meio ambiente. Da produção de ferramentas para facilitar a caça nas tribos nômades, o homem, mais do que nunca, começa a modificar o ambiente quando fixo. Agora não era mais necessário que o animal se adaptasse; o ambiente se torna adaptável.

O uso de agrotóxicos vem mais uma vez da necessidade do homem de se adaptar, nessa luta constante com o ambiente, para que sempre continue soberano. Para isso, substâncias químicas foram desenvolvidas nas mais diversas formas: pós, grãos, soluções, são pulverizados no ambiente.

Os biocidas usados no combate a insetos e outros animais indesejados, comumente chamados de praga, podem possuir diversos mecanismos de ações, como aqueles que afetam a respiração celular, baseados em arsênicos, ou ainda os raticidas anticoagulantes. Aqui será descrito apenas os grupos químicos mais comuns utilizados como inseticidas, tanto no uso agrícola, como nas políticas sanitárias no combate a vetores no meio urbano e no uso doméstico, que possuem ação sobre o sistema nervoso central (SNC). Eles são classificados conforme a família química da molécula, sendo organoclorados, organofosforados, carbamatos e piretroides (MORAGAS & SCHNEIDER, 2003).

O primeiro organoclorado identificado como agrotóxico foi o DDT, em 1940, quando Paul Mueller, da companhia suíça GEISY, observou que ele, sintetizado em 1874, era um potente inseticida. Essa classe de agrotóxico possue um grupamento químico com cloro que impede o fechamento dos canais de sódio na membrana celular dos neurônios, interferindo na repolarização da membrana. Isso faz com que o neurônio esteja sempre excitado, com uma superestimulação do sistema nervoso central e periférico. A toxicidade aguda dos organoclorados é bastante baixa, cativando a tranqüilidade da população no seu uso. Porém a sua alta solubilização em gorduras e sua baixa degradação natural faz com que ele fique acumulado nos tecidos. Além disso, por precisar uma concentração cada vez maior para o defeito desejado, o uso e o passivo ambiental desses produtos contribuíram com uma grande importância na contaminação ambiental. A exposição crônica do indivíduo a esse tipo de químico pode resultar em altas concentrações de acumulação no organismo. E ainda, pela característica de ser muito lipossolúvel, o processo de bioacumulação, durante o fluxo trófico, é bastante acentuado. Tem-se mostrado que esses produtos possuem potencial carcinogênico e teratogênico (FLORES *et al*, 2004).

A ação dos piretróides se assemelha aos organoclorados. Eles também agem evitando a repolarização da membrana neuronal, gerando um desequilíbrio do fluxo do sinal elétrico. Embora os piretróides não possuam a capacidade de se acumular, eles possuem uma alta toxicidade aguda.

O uso de agrotóxicos piretróides na agricultura começou na década de 1970, sendo muito recente. Não há muitas informações toxicológicas sobre o efeito dessa classe (SANTOS *et al*, 2007).

Os organofosforados e carbamatos possuem o mesmo mecanismo de ação: ambos são inibidores da acetilcolinesterase, chamados de anticolinesterásicos. Ambos possuem uma alta degradação no meio ambiente, sendo os carbamatos mais instáveis que os organofosforados. Quando ligados à enzima, formam complexos de inibições diferentes, os carbamatos carbamoilam o sítio ativo, enquanto que os organofosforados formam um complexo por fosforilação. O complexo do sítio fosforilado origina uma ligação forte e estável, sendo um inibidor irreversível. Já o complexo carboxilado é facilmente hidrolizado, podendo a enzima ser restaurada na sua forma ativa. Porém os carbamatos possuem uma afinidade muito maior pelo sítio ativo da AchE, fazendo com que sua ação seja mais rápida. A ação direta sobre essa enzima torna esses produtos muito eficientes. Primeiramente os organofosforados foram desenvolvidos na indústria bélica na II Guerra Mundial, como arma química, após foram utilizados no controle de “pestes” na agropecuária. Os carbamatos, por serem menos estáveis e com maior velocidade de ação e degradação, foram desenvolvidos e incentivados por serem mais “seguros” que os organofosforados. No Brasil, o uso desses químicos é bastante significativo e há 37 ingredientes ativos organofosforados e 18 carbamatos registrados. (ANVISA, 2009; MOSER, 1995; BARON, 1994)

A AchE é uma enzima componente das sinapses nervosas e junções neuromusculares, responsável pela hidrólise do neurotransmissor acetilcolina. A AchE também é importante nas expressões não-sinapticas, não-colinérgica, encontradas no desenvolvimento de células hematopoiéticas e neuronais, na regeneração neuronal e também em doenças neurodegenerativas como a Doença de Alzheimer (AD) (JOHNSON & MOORE, 2000). A transmissão do impulso elétrico nas conexões neurais e neuro-musculares ocorre através da liberação do neurotransmissor Ach na fenda sináptica, provocando a despolarização da membrana do neurônio receptor ou a contração muscular. Assim a liberação da Ach na fenda sináptica ocorre através do potencial de

ação quando alcança o botão terminal de um neurônio pré-sináptico, abrindo um canal de cálcio. Com a entrada de íon de cálcio, Ca^{2+}, ocorre a exocitose de vesículas pré-sinápticas contendo Ach, liberada então na fenda sináptica. Para que ocorra a repolarização da membrana, é necessário que essa Ach seja hidrolisada através da AchE ancorada na membrana plasmática (KOHILA, 2004).

2.2. O USO DE ANIMAIS NA PESQUISA CIENTÍFICA

Para o objetivo de predizer os efeitos tóxicos das substâncias, desde a sua origem, a toxicologia vem se utilizando de animais para mimetizar os efeitos em humanos. Hoje, os testes toxicológicos em animais, por não possuírem substitutos não-animais, estão incorporados em todas as Farmacopéias e em leis de fiscalização e regulamentação de produtos químicos.

Ao mesmo tempo em que os testes em animais possuem grande credibilidade e compõem um dos pilares do conhecimento científico, tanto básico, como aplicado à segurança em saúde e meio ambiente, é visível a crescente problematização que as práticas de pesquisa envolvendo animais vêm sofrendo, não apenas a partir da sociedade civil, como também de setores acadêmicos. Essa problematização vem ancorada ao avanço dos pensamentos filosóficos sobre as considerações morais aos animais e também nas falhas de extrapolação dos dados obtidos em animais não-humanos para humanos.

2.2.1. Limites éticos

> Todo o científico serve pelo menos dois deuses que, ao longo da história da ciência e até hoje, lhe pareceram absolutamente complementares. Hoje, devemos saber que eles não são somente complementares, mas também antagonistas. O primeiro deus é o da ética do conhecimento, que exige que tudo seja sacrificado à sede de conhecer. O segundo deus é o da ética cívica e humana.

> O limite da ética do conhecimento era invisível, *a priori*, e nós transpusemo-lo sem saber; é a fronteira para lá da qual o conhecimento traz com ele a morte generalizada: hoje, a árvore do conhecimento científico corre o risco de cair sob o peso dos seus frutos, esmagando Adão, Eva e a infeliz serpente. (MORIN, 1994, p. 30)

Foi a partir das sentenças do Tribunal de Nuremberg, publicado pelo Nuernberg Military Tribunals em 1949 em uma seleção de casos médicos no *Trials of War Criminals before the Nuernberg Military Tribunals*, que foi proclamado o Código que dispõe de diretrizes éticas internacionais para pesquisa com humanos. O primeiro princípio estabelecido, retirado do documento já citado (p. 181)

> "O consentimento voluntário do sujeito humano é absolutamente essencial. Isso significa que as pessoas que serão submetidas ao experimento devem ser legalmente capazes de dar consentimento; essas pessoas devem exercer o livre direito de escolha sem qualquer intervenção de elementos de força, fraude, mentira, coação, astúcia ou outra forma de restrição posterior; devem ter conhecimento suficiente do assunto em estudo para tomarem uma decisão. Esse último aspecto exige que sejam explicados às pessoas a natureza, a duração e o propósito do experimento; os métodos segundo os quais será conduzido; as inconveniências e os riscos esperados; os efeitos sobre a saúde ou sobre a pessoa do participante, que eventualmente possam ocorrer, devido à sua participação no experimento".

Assim os voluntários devem ter condições de entender a pesquisa e os riscos. Segundo esse artigo, deve-se excluir da pesquisa todas as pessoas em situação de risco social, menores de idade, e pessoas sem juízo pleno.

A sociedade, através dos casos de experimentação com humanos por parte dos pesquisadores nazistas divulgados após a Segunda Grande Guerra, aprendeu que a ciência não está acima dos limites da ética cívica, da igualdade e da justiça, por mais que fossem evidentes na nossa história outros casos de experimentação com humanos. São exemplos ocorridos nas pesquisas estadunidenses o caso do Estudo de Tuskegee que, entre os anos e 1932 a 1972, negou informações

sobre a doença e sobre o tratamento a 399 homens negros com sífilis (COBB, 1973). E ainda o caso de experimentação com humanos, contemporâneo ao Tribunal de Nuremberg, divulgado durante o pedido de desculpas do atual presidente dos Estados Unidos, Barack Obama em 2010 para a Guatemala (TANNE, 2010). Nesse estudo, de 1946 a 1948, foram contaminados com sífilis 696 guatemaltecos, entre prostitutas, prisioneiros e internados em hospitais psiquiátricos, para verificar o efeito da penicilina logo após a relação sexual.

Singer (2000, 2007), baseando-se na ampliação dos critérios morais de Bentham (2007), da capacidade de pensar para a capacidade de sentir – ou senciência, é um dos maiores expositores sobre ética animal. Este autor defende que um interesse, ao existir, deve ser respeitado, independente da qualidade do portador desse interesse. No momento em que os animais são capazes de sentir e possuem o interesse em não sentirem dor, ou privação de liberdade, gera uma obrigação por parte dos humanos de proteger esses interesses. Além disso, é clara a comparação entre o especismo (a desconsideração do interesse de seres de outras espécies para a valorização de interesses menos importantes de humanos) e outros preconceitos não mais aceitos na cultura ocidental, como o sexismo (a desconsideração do interesse das mulheres em relação a interesses menos importantes dos homens) e o racismo (desconsideração do interesse de pessoas de outras etnias).

Assim, foram identificados já no século passado os limites impostos à ciência no que se refere ao uso de seres humanos em pesquisa. Porém, com o avanço das discussões em ética aplicada aos animais, podemos identificar outra fronteira da ética científica, onde ainda existe a morte generalizada de animais sencientes.

2.2.2. Novo Paradigma Científico: A Substituição do Modelo Animal

Dois dos critérios atuais da toxicologia são a redução e a substituição do uso de animais em testes, gerando estudos sobre biodisponibilidade e toxicocinética de substâncias, observações de novos efeitos adversos, melhorando os testes e entendendo melhor os mecanismos de ação dessas substâncias tóxicas (MEYER, 2003).

Estudos de validação para testes toxicológicos já estão ocorrendo devido a essa pressão da sociedade e das autoridades governamentais, apoiado pelo governo britânico e da Comissão Européia desde o final do século passado, como o estudo de validação de métodos alternativos ao Teste Draize de irritação ocular, no qual uma substância é colocada em um dos olhos de coelhos para ser analisado o grau de irritabilidade (BALL *et al*, 1995).

Esse apoio ao desenvolvimento de métodos alternativos foi incorporado na Legislação REACH (Registration, Evaluation and Authorisation of Chemicals) da União Européia. A legislação REACH, além de incentivar a produção de métodos alternativos ao uso de animais em toxicologia, incentiva a produção de modelos de extrapolação dos dados *in vitro* para a atividade tóxica da substância, através de dados de toxicocinética (COMBES, 2008). Essas informações são importantes para entender o efeito e o comportamento do toxicante através de uma visão sistêmica.

O novo paradigma em toxicologia baseado em pesquisas não-animais diminuirá os gastos nas pesquisas (ver quadro 1), utilizando menos material e analisando as perturbações de respostas celulares, no lugar de desfechos em animais com parâmetros toxicológicos.

QUADRO 1. Opções para futuras estratégias de testes de toxicidade

Opção I *In vivo*	**Opção II** *In Vivo* **com Bem-estar animal**	**Opção III** *In Vitro* **e** *In vivo*	**Opção IV** *In Vitro*
Biologia animal.	Biologia animal.	Primariamente	Primariamente

		biologia humana.	biologia humana.
Doses altas.	Doses altas.	Diferentes doses.	Diferentes doses.
Baixa produção.	Produção aumentada.	Média a alta produção.	Alta produção.
Caro.	Menos caro.	Menos caro.	Menos caro.
Consumo de tempo.	Menor consumo de tempo.	Menor consumo de tempo.	Menor consumo de tempo.
Uso de grande número de animais.	Uso de menos animais.	Uso de substancialmente menos animais.	Praticamente sem uso de animais.
Baseado em desfechos extremos.	Baseado em desfechos extremos.	Baseado em perturbações de respostas celulares críticas.	Baseado em perturbações de respostas celulares críticas.
	Algumas varreduras usando abordagens computacionais e *in vitro*; maior flexibilidade que os métodos tradicionais.	Varreduras utilizando abordagens computacionais possíveis; limitados estudos em animais que se concentram no mecanismo e metabolismo.	Varredura utilizando abordagens computacionais.

*Adaptada de CTTAEA, 2007

Para essa mudança de paradigma, é necessário uma interdisciplinaridade de conhecimentos, aplicando os conhecimentos adquiridos *in vitro* sobre os toxicantes, através da análise das perturbações celulares em cultura de células expostas a certa dose de toxicantes (ver figura 1), com ferramentas computacionais de modelagem de resultados, chamados métodos *In silico*, associados a uma melhor compreensão dos dados coletados e disponíveis, através de revisões críticas da literatura e evitando a reprodução desnecessária de experimentos (CTTAEA, 2007).

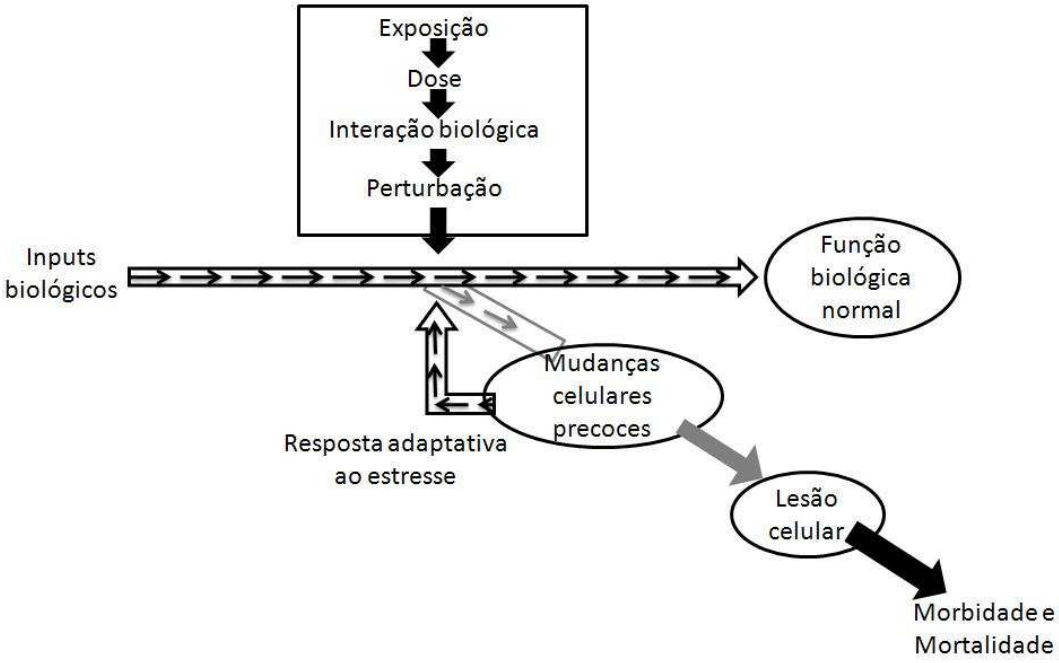

FIGURA 1. Mudança de paradigma toxicológico para análise de perturbações celulares como desfechos mais sensíveis. Adaptado de CTTAEA, 2007

2.4. LINHAGEM CELULAR DE NEUROBLASTOMA HUMANO (SH-SY5Y)

Para o desenvolvimento da metodologia para avaliação de anticolinesterásicos desenvolvida na terceira parte dessa dissertação foi utilizada uma linhagem de neuroblastoma humano (SH-SY5Y). Essa linhagem é originária de um processo triclonado (ver quadro 2) a partir da linhagem SK-N-SH, estabelecida em cultura em 1970, a partir da biopsia da metástase de um neuroblastoma na medula óssea de um humano (*Homo sapiens*) do sexo feminino de 4 anos de idade e depositada na *American Type Culture Collection* (ATCC) por JL Biedler. (ATCC, 2011; BIEDLER *ET AL*; 1978).

QUADRO 2. Derivação das linhagens celulares de neuroblastomas humanos

Linhagens	**Descrição**
SK-N-SH	Linhagem de neuroblastoma não-clonado cultivado em Dezembro de 1970.
SH-SY	Sublinhagem clonal de SK-N-SH com características de neuroblasto.

SH-SY5	Subclonada a partir da linhagem SH-SY
SH-SY5Y	Subclonada a partir da linhagem SH-SY5
SH-IN	Clone da linhagem SK-N-SH com características de neuroblasto
SH-EP	Clone da linhagem SK-N-SH com características epiteliais.
SH-FE	Clone da linhagem SK-N-SH com características epiteliais.
SK-N-BE (1)	Linhagem de neuroblastoma não-clonado cultivado em Junho de 1972.
SK-N-BE (2)	Linhagem de neuroblastoma não-clonado cultivado em Novembro de 1972.
SK-N-MC	Linhagem de neuroblastoma não-clonado cultivado em Setembro de 1971.
MC-IIE	Sublinhagem biclonada de SK-N-MC
MX-IXC	Sublinhagem biclonada de SK-N-MC

Adaptado de Biedler *et al* (1978).

A linhagem SK-N-SH possui dois tipos celulares distintos: um com células densas e pequenas, com citoplasmas escassos, formando agregados e outro com células relativamente grandes e epitelióides (Biedler, 1973).

A linhagem SH-SY5Y é usada como um modelo *in vitro* de neurônios dopaminérgicos para testes de neurotoxicidade. Quando indiferenciada, elas são neuroblastomas imaturos que mantém algumas características de células tronco, com proliferação agressiva. Mesmo indifenciadas, elas expressam a AchE na membrana celular. Porém, para outros testes de expressão da enzima e análise de outras vias de toxicidade, pode-se induzir a diferenciação celular através de ácido retinóico (AR) combinado com outros fatores de crescimento, como BDNF (Brain-derived neurotrophic factor), para cessar a fase proliferativa e desenvolver as características dopaminérgicas e neuronais, como emissão de neuritos (LOPES *et al*, 2010; SONG & LUCHTMAN, 2010; RADIO & MUNDY, 2008; ENCINAS *et al*, 2000).

3 – OBJETIVOS

3.1. GERAL

Analisar o uso de cultura de células de linhagem dopaminérgica (SH-SY5Y) como método para determinar o potencial inibitório de anticolinesterásicos.

3.2. ESPECÍFICOS

Descrever sistematicamente a literatura científica mundial sobre métodos alternativos ao uso de animais em testes toxicológicos de agrotóxicos, descrevendo a sua distribuição por áreas.

Analisar o potencial uso de cultura de células viáveis na determinação e acompanhamento do potencial anticolinesterásico de agrotóxicos, usando como teste o carbamato Aldicarb.

4 – MATERIAIS E MÉTODOS

Este trabalho se propôs a um estudo sistemático da produção científica mundial e a estabelecer uma metodologia *in vitro* alternativa ao uso de animais para análise de anticolinesterásicos, testando um pesticida carbamato de alto impacto na saúde pública, a fim de facilitar e possibilitar maior flexibilidade para os testes de regulamentação, fiscalização e toxicidade de agrotóxicos e outras substâncias com igual mecanismo de ação. Nesse sentido, os procedimentos metodológicos estão descritos, detalhadamente, nos artigos científicos, forma pela qual foi estruturada a presente dissertação. Ambos os artigos estão no padrão para publicação na revista PlosONE (Public Library of Science), sendo o primeiro configurado no modelo de Revisões Sistemáticas, com tabelas maiores que uma página e figuras separadas como anexos após o referencial teórico, e o segundo distribuído seguindo o modelo de publicação geral.

5 – RESULTADOS

5.1. ARTIGO 1: What Do We Know About Alternative Methods to Pesticide Toxicology? A Systematic Review.

Este espaço foi deixado em branco propositadamente.

What Do We Know About Alternative Methods to Pesticide Toxicology? A Systematic Review.

Abstract

The use of pesticides to control living organisms that threaten human health and crops/food supply is continuously increasing. Conflicting with that, there are several evidences that such increase may pose a danger to environmental and human health. In order to achieve proper regulation of pesticide use and exposure, several toxicological tests are available employing animal testing. However, the extrapolation of the results obtained from such species to humans and other non-target species is still a matter of intense debate. In the meantime, a large amount of animals is used in toxicological tests worldwide, reinforcing the ethical imperative to develop final alternative methods to these tests. In this context, the aim of this study was to systematically review the scientific literature about alternative methods to pesticide toxicity assays. Embase, LILACS, IBECS, MedCarib, Wholis, Web of Science and Scielo were searched until April 24, 2010, by means of several databases. The search on MedLine by PubMed used the following Medical Subject Headings (MeSH): "Animal Use Alternatives" with the terms related to "pesticid*". Search of scientific literature returned 287 abstracts, which were analyzed and categorized as "off-topic", "review", "regulations", "reduction and refinement" and "replacement". After all exclusions, 37 articles were selected to be reviewed. Among them, 10 were classified in off-topic study and 27 articles were analyzed and classified in 8 scientific fields. 55% of the articles were classified in acute toxicity (LD 50) or eye irritation, the two more explored fields. Therefore, this systematic review showed that only few studies issue alternative methods to the use of animals in pesticide toxicological tests and they are concentrated in only two fields. Other fields can be explored by new methods. Research on replacement methods needs to be encouraged as it could lead to significantly reduce the current massive use of animals in experimental science.

Introduction

...and there was the girl. She was happy to participate in the International Wine Exposition. She decided to sample the wine from a developing country, sure that production of quality wine was a great economical alternative. Moreover, she could drink local products anytime and she would like to experience different flavors from other places, which she possibly would never visit. As she was

in line to taste the foreign product, she almost could touch the exotic and bucolic image of the crushing of grapes, imagining the festival of picking for a new cycle of the wine. While the girl still waited for her time in that afternoon, the worker was coming home at the evening, after an exhaustive day in a grape farm. He had been contracted only to apply the pesticides at the vineyard, for as much time as necessary. He got to his family and hugged his children, still afraid for the strong dizziness and nausea felt a few moments before. Just wishing for this day to end, he did not even care to change his worksuit before entering his house.

This is a fictional story; however, this is the image of many agricultural works around the world, especially in developing countries, where the use of pesticide is increasing in response to many problems of health and crop/food production [1,2]. The exposure to these chemicals, possibly on a daily basis, can be occupational, particularly in agricultural work, where about 85% of the pesticide used in the world, but also through public policies of vector disease control, responsible for other 10% of the world´s pesticide usage [3]. Indirect exposure may also occur by the consumption of treated foods [4] and in peculiar situations, such as the carrying of pesticides in worker´s vestures or the domestic use of insecticide.

Due to the risks to human health and to the environment offered by those chemicals, the regulatory aspects of their use rely on exhaustive studies on their toxic effects, which is done often through animal testing. The use of animal models in toxicology has been criticized in all social fields and a new toxicological paradigm have been developing, focusing on the alternatives to animal testing [5], based on the 3R (reduce, refine and replace) concept [6]. Therefore, this review has the intention of analyze the scientific production to identify studies with this concern on topics related to pesticide toxicology. Several data basis were searched through the use of different keywords cross-related with the term “alternatives to animal testing”. After refining the consult, were identified 26 studies focused on alternative methods to toxicological tests applied to pesticides, the majority issuing acute toxicity or eye irritation.

Methods

Searching

The databases and databanks MedLine, Embase, Lilacs, IBECS, MedCarib, Wholis, Web of Science (WoS) and Scielo were searched until April 24, 2010,, in English, Portuguese and Spanish, depending on the search engine main idiom. The search on MedLine by PubMed used the following Medical Subject Headings (MeSH): “Animal Use Alternatives” with the radical keyword

“pesticid*”. Web of Science, Scopus and Lilacs, were searched in the Topic section (issuing title, abstract and keywords) crossing radical terms: “alternativ* and animal* and method*” with “pesticid* or insecticid* or fungicid* or molluscacid* or rodenticid*”. To prevent bias of misclassification in Lilacs, keywords were used to find references, instead of specific descriptors. In other Latin databases it was used identical word radicals in Portuguese, Spanish and English in the *title*, *abstract* and *keyword* fields, crossing “pesticid$ or insecticid$ or fungicid$ or molluscacid$ or rodenticid$” and “alternativ$ and anim$”.

Selection

All abstract and titles found by the described search strategies were screened and categorized as “off-topic”, “review”, “regulations”, “reduction and refinement” and “replacement”. Were considered "off-topic" those articles dealing with alternative methods to pest control, those aiming to improve animal tests themselves or those that didn’t test pesticides. Articles assigned as “reduction and refinement” and “replacement” were analyzed thoroughly and once again classified by aims, chemical tested and method used.

Data abstraction

All analyzes were done by two revisers (RB and ES) independently, using data formularies to excerpt aims, methods, target organism, tested substances and conclusion of the articles, making a critical conclusion of the articles and marking due toxicological field, 3 R’s concept and conclusions. Articles on the same field and issuing the same pesticides were grouped to detail analysis. Discordant classifications were analyzed by a third researcher (AM).

Quantitative data synthesis

This review is primarily a descriptive work to select, within the scientific literature, methods which can be used in the assessment of pesticide and to promote directives to new research in alternatives to pesticide toxicology. However, several articles were found issuing eye irritation assessment using the same method of analysis, comparing in vitro and in vivo results. Therefore, for this specific class it was possible to group the results and to do a general comparison.

Results

The described search strategy of scientific literature returned 287 abstracts (126 from Scopus, 77 from WoS, 36 from PubMed, 39 from Lilacs, 5 from IBECS, 2 from Scielo, 1 from MedCarib and 1 from Wholis). MedLine was consulted from three distinct databases (Scopus, PubMed and WoS) which returned many triplicates and duplicates: 5 triplicates in Scopus, PubMed and WoS; 16 duplicates in Scopus and PubMed; 5 in Scopus and WoS; 1 duplicate in LILACS and Scielo and in LILACS and WoS. Other 186 abstracts were also excluded as they were considered "off-topic" and 20 because they were reviews, while 11 focused on regulatory issues. In this manner, after all exclusions, 37 articles were selected to be reviewed (Figure 1). Among them, 11 were considered off-topic studies. All the other studies were further classified in accordance with the table 1.

Acute toxicity studies

The studies on alternative tests to acute toxicity found in this review concentrate in two distinct fields: strategies for the reduction on animal use by estimating of LD50 and tests on other target organisms (cell culture, yeast and nematode).

To classify the lethality of pesticides by estimating the LD50, two different models were found: Acute toxic class (ATC) method [8,9] and an improved technique of up-and-down procedure (UDP) by computer[7]. Both methods have the same stepwise classification procedure using fixed doses of 5, 25, 200, 2,000 and 5,000 mg/kg (EC, 2004).

Analyses on the model of LD50 were done in several studies. In order to analyze the usefulness of the nematode *Caenorhabditis elegans* as a model to acute toxicity [11], a correlation was done between EC50 (effective concentrations 50%) movement and LC50 (lethal concentration 50%) in *C. elegans* and LD50 in rats, to ten organophosphate pesticides (acephate, dimethoate, dichlorvos, dicrotophos, monocrotophos, methamidophos, phosphamidon, omethoate, phosdrin and trichlorfon). The ranking of *C. elegans* LC50 and EC50 showed significant correlation with the ranking of rat's LD50, indicating the validity of this alternative animal model to determine the toxicity. The use of the yeast *Saccharomyces cerevisiae* was also suggested to test acute toxicity [16]. In this study, 160 chemicals, including pesticides, were tested in the determination of the growth Inhibitory Concentration of 50% (IC50) and analyzing the correlations between yeast IC50 and rat LD50 (0.763) or mouse LD50 (0.728), and between rat and mouse LD50 (0.798). In

addition to this yeast model, a bacterial model employing *Bacillus stearothermophilus* was used to observe different toxic effects of the organochloride insecticide endosulfan (□ and □̃endosulfan isomers) on lipophilic compounds [Martins et al, 2003], describing a dependence between membrane effects and growth impairment which was used to generate an index of citotoxicity.

Interesting results of LD50 were also found with cell culture models. A study [13] employing a feline kidney cell line, found a good correlation between logarithmically transformed values of IC80, using for endpoints neutral red uptake (NRU), kenacid blue (KB) and highest tolerated dose (HTD) tests, with log rat DL50 to five substituted benzthioanilides (NRU = 0.826; KB = 0.800; HTD = 0.883). This model was used to evaluate the cytotoxic activity of the 12 substituted benzthioanilides potential fungicide substances using two commercial fungicides (thiuram and imaverol) as toxic references. Other study, intending to determine cell line specificity on the development of alternative methods, compared the action of two pesticides environmental pollutants (Pentachlorophenol and Rotenone) on three cell lines: HeLa (human adenocarcinoma), 3T3 (mouse embryonic fibroblasts) and Vero (green monkey kidney fibroblasts), describing Vero cells as more sensitive to reproduce toxic studies on chemicals [Freire et al, 2009]. The protective effect of the fetal bovine serum on the cellular sensibility to pesticides (Crabofuran, cypermethrin, lindane, glyphosate and 2,4-D) was also analyzed on other few works [14,15] on the hybridoma cell line 1E6. Morever, MTT assay and trypan-blue exclusion test showed a good correlation to analyze cytotoxicity with this cell line [15]. The authors concluded that a serum-free test would be more sensitive to evaluate pesticide-related cytotoxicity.

Ecotoxicity

Castaño and collaborators [17] wrote a rather complete report of the ECVAM workshop on the use of fish cells in Ecotoxicology. In that work, they aimed to make a critical review of the potential use of fish cells on replacement, reducing or refinement, discussing their advantages, limitations and future applications. Although the aim of the present work is to compile experimental studies, it was important to analyze and to divulge this report due it analyze an important method to ecotoxicity assessment. Specifically to pesticide toxicology the authors cited an article by Saito and collaborators of 1991, which failed to be retrieved according to our search strategy, that analyzed 45 pesticides. Distinct of other chemical classes, the correlation coefficient between EC50 and LD50 was poor to organophosphate (r = 0.69) and carbamate pesticides, whereas to non-classified pesticide it presented a r = 0.85. This difference occurs possibly due to the specific action

mechanism of organophosphate and carbamate in dopaminergic neurons. Castaño and collaborators [17] conclude, between other considerations, that fish cell methods offer many possibilities for improving hazard identification in environmental risk assessment, and they recommend a better characterization of the available fish cell systems, develop and standard protocols, in order to promote the application of these techniques to relevant compounds to ecotoxicology.

In vitro methods also are used to test toxic chemicals in the environment employing other aquatic organisms such as the marine bacterium *Vibrio fisheri* (bioluminescence inhibition), the green algae *Chlorella vulgaris* (growth inhibition), and microcrustacean *Daphnia magna* (acute toxicity immobilization test)[19]. Using this organism and tests, Zurita and collaborators [19] analyzed the toxicity of sodium fluoroacetate (SMFA), comparing these models with two fish cell lines (*Poecilliopsis lucida* hepatoma - PLCH-1 and rainbow trout - Oncorhynchus mykiss – RTG-2). The effects on PLHC-1 cells were measured using total protein content (Coomassie brilliant blue G-250), glucose-6P dehydrogenase leakage (G6PDH activity), neutral red uptake (NRU), mitochondrial activity (MTS tetrazolium reduction) and detection of apoptosis induction using TUNEL technology. To measure the effects on RTG-2 also were analyzed the acetylcholinesterase and ethoxyresorufin-O-deethlyase (EROD) activity. The authors found *D. magna* more sensitive to SMFA than other organisms. *C. vulgaris* also showed high sensibility, while *V. fischeri* presented low sensibility, possibly because SMFA blocks the citric acid cycle which does not interfere in facultative anaerobic bacteria. They conclude also that SMFA is most unlikely to produce acute deleterious effects to aquatic organisms, possibly because the three organisms used are much different on question of toxic mechanisms. PLHC-1cells were described as more sensitive to SMFA than the RTG-2, being the NRU the most sensitive bioindicator.

Zitova and collaborators [18] studied the respirometric screening technology (RST) assay for a bioindicator of ecotoxicity in *Escherichia coli* and *V. fischeri* (prokaryotes), brine shrimp *Artemisia salina* (microcrustacean), human lymphocyte Jurkat cells and embryos of zebrafish *Danio rerio.* They observed changes in the metabolic activity patterns in the presence of the pesticide lindane, as well as other chemical species (zinc, copper, pyrene and naphthalene), even though at different levels on an organism-dependent basis, concluding that, for chemicals with complex modes of action, animal-based assay require a longer time (24 h) of exposure. Cell-based assays (particularly those using E. coli) for assessment of acute toxicity have high initial sensibility. Eukaryotic cells (such as Jurkat) in spite of their reduced sensibility are expected to be the more relevant model for screening to human health.

Hepatotoxicity

The liver is often the site of production of metabolites, and a target organ for those same metabolites, as well as for xenobiotics. Studies on transformation of chemicals in liver are important to understand a systemic action of toxicants. Bull and collaborators [21] analyzed two genetically engineered murine NIH-3T3 cells, transfected with human CYP1A2 or CYP2E1 isoforms, and transfected Chinese hamster lung fibroblast V79 cells expressing rat CYP2B1 (all members of cytochrome P450 superfamily). Cytochrome P450 enzyme expression is important in phase I, responsible for the metabolism of xenobiotics, catalyzing their oxidation, reduction or hydrolysis reaction, making the resulting molecules available to phase II reactions, where functional groups previously added will be bind to hydrophilic groups. Therefore, some drugs may be bioactivated in those phases, resulting in metabolites more pharmacologically or toxicologically activated. In Bull and collaborators (2001), cell activity was assayed by NRU and Alamar Blue reduction, while oxidative stress was assessed by measuring glutathione (GSH), following the exposure to 1,3-dichloropropanol (DCP) or cyclophosphamide (CPA). The authors concluded that all engineered cells tested are able to show metabolism-mediated toxicity following exposure to DCP and CPA, even though NRU and Alamar Blue assays presented limitations in evaluating hepatic metabolism-mediated toxicity, detecting just the initial stages of cytotoxicity..

At 1995, Montesissa, Lucisano and Carli also studied the metabolism-mediated toxicity [22]. The authors analyzed the Aldicarb (ALD) metabolism using hepatic microsomes from chickens, rabbits, sheep and pigs. The sulfide group on ALD structure is rapidly metabolized to sulfoxide (ALDSOX), which is more effective to inhibit acetylcholinesterase. Posteriorly, the sulfoxide is converted to the less toxic sulfone (ALDSON). From the four species studied, rabbit showed the highest concentration of CYP450 in hepatic microsomes, followed by sheep. Sheep microsomes , on the other hand, had the greatest overall oxidative activity. It was also observed a rapid attainment of steady-state concentrations in pigs and sheep and a relation between the CYP450 content and the ALDSON production on both species, but this correlation was not found to the ALDSOX. These data demonstrate the utility and necessity of *in vitro* studies on metabolism-mediated toxicity.

It also was developed a battery of screening tests on hepatotoxicity[20]. Three targets were employed: freshly isolated rat hepatocytes, primary cultured rat hepatocytes, and rabbit eye derived cells (SIRC cells). Seven organophosphates (fenthion, diazinon, disulfoton, edifenfos, phenthoate, IBP, trichlorfon), two S-triazines (prometryn, ametryn), seven carbamates (diethofencarb,

mefenacet, benfuracarb, swep, ethiofencarb, isoprocarb, alanycarb), two phenylureas (linuron, cinosulfuron, thifensulfuron methyl), two pyrethroids (permethrin, cyfluthrin), four diphenylethers (chlornitrofen, nitrofen, chlormethoxyfen, bifenox), and aramite were tested for cytotoxicity (LDH leakage method), cellular glutathione levels (phase II enzyme activity) and ethoxycoumarin O-deethylation (ECOD) and ethoxyresorufin O-deethylation (EROD) activities (phase I enzymes). Based in the results, the pesticides were classified in four groups: (1) cytotoxic to both isolated hepatocytes and primary cultured hepatocytes (several compounds demonstrated hepatotoxicity at lower concentrations than on SIRC cells, suggest liver-specific toxicities); (2) inducing decreased viability of primary cultured hepatocytes but not freshly isolated hepatocytes (authors suggested that sustained decrease in GSH in primary cultured hepatocytes may exposure the cells to oxidative stress and/or to toxic metabolites); (3) changes could not be found in the cell viability of both hepatocytes, but there were decreased of ECOD activity; and (4) inducing an increase on ECOD activity by a lower concentration than cytotoxic dose. The authors suggested that this battery of *in vitro* tests is useful to screen the hepatotoxicity of pesticides.

Eye irritation

Two methods were found in the present search evaluating the eye irritation caused by pesticides: Chicken Enucleated Eye Test (CEET) and Hen Egg Chorioallantoic Membrane (HET-CAM) bioassay.

The chicken enucleated Eye test (CEET) or Isolated Chicken Eye (ICE) test was developed by Prinsen and Koëter (1993) from the Isolated Rabbit Eye (IRE) test by Burton et al. (1981). It is an *ex vivo* test, using freshly isolated chicken eyes (obtained from slaughterhouses) to evaluate the potential ocular irritancy, corneal swelling, corneal opacity and fluorescein retention (ECVAM DB-ALM, 2010). Prinsen [29] applied this method to evaluate the ocular irritation potential of 44 test materials received in TNO Nutrition and Food Institute, 4 of those being pesticides. The correlation coefficients between rabbit *in vivo* and CEET tests to all materials was very high (0.92 to 1 h of interval). Of the 44 tested substances, 7 showed different or borderline classifications between CEET and *in vivo* rabbit, including 2 of the 4 tested pesticides.

The HET-CAM test is based in irritant endpoints (hyperemia, hemorrhage and coagulation) on the vascular fetal membrane of chicken embryos, known as the chorioallantoic membrane (CAM), to estimate the effects of substances on the conjunctiva of the eye (ICCVAM, 2010). 6

articles were found using this method [23,24,25,26,27,28]. All of them presented a comparison with *in vivo* eye irritation test, either tested or reported from other references. Table 2 presents the chemical tested and the results from such studies. Four pesticides were also tested (Silwet L-77, Kohinor 200 SL, Previcur 607 SL and Confidor 200 SL) on mitochondrial metabolism on VERO cell line, using the MTT assay, but no statistical test of correlation was presented[25].

Eye irritation - Quantitative data synthesis

From HET-CAM studies, it was possible to do a global comparison, standardizing the toxic classification from *in vitro* and *in vivo* results presented in the articles described in Table 2. Classification of HET-CAM and *in vivo* results were standardized to four categories: (1) Not labeled, (2) Slight irritation, (3) Moderate irritation and (4) Severe irritation. To the HET-CAM results, it was used the irritation score (IS), except the results of one study [24] which employed the authors own classification. These results were standardized according to the classification presented on table 3.

Table 3. Standardized categories

Standard classification	**HET-CAM IS [23,24,25,26,27,28]**	**HET-CAM [24]**	**In vivo classification**
Not labeled	0 to 0.9	Nonirritant	Non-irritant; no irritation.
Slight irritation	1 to 4.9	Weak irritation	Mildly irritant C2A; minimally; mild irritation; slight irritation; weak irritation.
Moderate irritation	5 to 8.9	Moderate	Moderate; irritation.
Severe irritation	9 to 21	Irritant	Eye irritant C1; irritant; extremely; extremely irritation; severe; extreme.

In a general analyze between *in vitro* and *in vivo* valuation, it was found a concordance classification to 19 pesticides (59.4%). Only to 3 substances in vitro test underestimated the hazard irritation (9.3%) and to 10 (31.25%) chemicals, the in vitro method overestimated the irritation potential (Figure 2). The overestimation possibly happens due to the in vitro test exhibit a higher sensibility than *in vivo* test. Moreover, the overestimation of the hazard don't invalidate the method

for the purpose of health protection. It is necessary more studies specially on the pesticides presented in the underestimated hazard zone.

Skin permeability

The research strategy employed returned only one article investigating the use of alternative methods to skin permeability to pesticides, comparing in vitro and in vivo methods under identical experimental conditions [30]. This work compared *in vivo* tests using rats and human volunteers to three *in vitro* models, namely rat skin membranes, human skin membranes and a perfused pig-ear model, after exposition to Propoxur ((2-isopropoxyphenyl) N-methyl carbamate). The authors observed that the epidermal membranes were more permeable to propoxur than viable skin membranes, and that the pesticide is partially retained or deposited in the dermis and is only slowly released into the receptor fluid. Comparing the *in vivo* skin permeability test using rat and human models , it was found that rat skin is more permeable than human skin, since it attains the peak blood concentration after 0.5 h, as compared to aprox. 7 h after administration for human skin. Authors considered that the *in vivo* rat model overestimates human skin absorption of propoxur and that the data obtained indicate that the *in vitro* models could be very useful to estimate human risk, even though these tests also should be performed with lipophilic penetrants.

Neurotoxicity

The major action mechanism of carbamate and organophosphate pesticides is the inhibition of acetylcholinesterase (AchE)[33]. This important enzyme catalyzes the hydrolysis of the neurotransmitter acetylcholine and its inhibition is used as an endpoint to neurotoxicity tests. Rajini and collaborators [11] analyzed the correlation between AchE inhibition and reduction in movement and mortality in the worm *Caenorhabditis elegans* after exposure to 10 organophosphates (Acephate, dimethoate, dichlorvos, dicrotophos, monocrotophos, methamidophos, phosphamidon, omethoate, phosdrin and trichlorfon). The authors found a reduction in AchE activity even at the lowest concentration tested, and a significant inhibition in Ache activity even in pesticide exposure concentrations not effecting changes in behavior or in lethality. Nevertheless, due to difficulties inherent to the measuring of AchE activity, it is considered more reliable and easier to use LC50 and movement endpoints to determine the toxicity of organophosphate pesticides.

Reproductive toxicity

In order to assess reproductive toxicity, Lazzari and collaborators[31] on a study from the European ReProTect project, used bovine germ cells, developing a test of oocyte maturation and *in vitro* fertilization. The test consists of the use of immature oocytes from bovine ovaries (obtained from from slaughterhouses) and, after treatment with the test chemicals, analyze their effects through the evaluation of the completion of meiosis up to the metaphase II stage. Additionally, in the maturation test, matured oocytes were coincubated with frozen-thawed bovine semen and posteriorly exposed to the test chemicals, in order to evaluate the formation of female and male pronuclei, obtaining the percentage of fertilized oocytes. With such methodologies, Lazzari and collaborators[31] assayed, among other chemicals tested, the reproduction toxicity of 6 pesticides and agricultural chemicals: cadmium chloride, carbendazim, cycloheximide, diethylstilbestrol, lindane and methyl acetoacetate. The authors suggest that this tests, combined with other models and battery tests, are able to predict chemical hazards on mammalian fertility.

Adrenotoxicity - endocrine disruptors

Aiming to analyze the potential use of an *in vitro* cell model to test action of endocrine disruptors on adrenal steroidogenesis, Oskarsson and collaborators[32] described the expression of nine genes involved in the steroidogenic pathway and the cortisol secretion on human adrenocortical carcinoma cell line H295R, exposed to the organoclorine pesticide Lindane. Before the exposition to Lindane, the cells were exposed to forskolin, in order to promote cellular differentiation, observing the gene expression pattern more similar to that in the human adrenal gland. This work showed the effects of lindane on adrenal cortisol secretion and gene expression *in vitro,* suggesting that this cell line (H295R) and the methodology employied can both be used successfully to test the modulation of hormone production and gene expression by adrenotoxic endocrine disruptors, being an important screening tool for studies on environmental adrenotoxicants and on therapeutic drugs with adrenal side-effects.

Discussion

The present work assessed the up-to-date relevant scientific literature on alternative methods to pesticide toxicity evaluation through a intentionally wide-ranging search strategy, in order to include as much articles as possible. Nevertheless, after our classification on relevancy and novelty,

defined by the introduction of new methods or modifications on existing ones, led to the more profound critical reading and description of only about 10% of the records initially found. Also, as expected, due to the searching being done in diverse databases, many duplicates (23) and several triplicates (5) were found.

This review only searched for studies which declaredly aimed to introduce alternatives to animal testing (refine, reduce or replace animal use) and, therefore, numerous studies on *in vitro* toxicology, with possible relevancy to the theme, were not found by the search strategy as they didn't register that objective. We became aware of this issue by consulting the bibliography of some reviews on alternative methods, which escaped from our search (data not found). It demonstrates that the authors should to attempt this developing study field and insert this theme in their work-keys and topics.

The filtered and refined results of the initial systematic search showed that there are only few studies about alternative methods to the use of animals in pesticide toxicology and that the explored fields strongly concentrate on acute toxicity and eye irritation; possibly this interest reflects advances on the available toxicological knowledge and methodologies on these areas, motivated by the pharmaceutical and cosmetics Industry.

On acute toxicity, UDP was validated to reduce and refine animal use, replacing the LD 50 test [34]. This decision was accepted by many American control agencies, including the Agency for Toxic Substances and Disease Registry (ATSDR)[35], the Food and Drug Administration (FDA)[36] and Environmental Protection Agency (EPA)[37]. However, this method can't replace the use of test animals, and its contribution is really important and needs to be divulged mainly in countries where Alternative Methods are not a scientific or regulatory issue. Moreover, new methods that aim to replace animal use need to be better explored, as a yeast model for acute toxicity on *S. cerevisiae* IC50 showed levels of correlation with rat and mouse LD50 which are similar to those observed between the two murine groups [16]. It raises the hypothesis that the correlation between yeast and animal models is as different as human and other animals. Therefore, registries of human epidemiology data need to be used in comparisons of effects on human and other organisms.

Additionally, these differences of chemical metabolism among different target organisms can have an effect on the choice of target, such as a different liver metabolism of pesticide [22]. This difference orients to use, in studies on human health, human tissues or cells, such as human hepatocyte cell lines. The interspecies differences need to be evaluated prior to the development of

a new method. Moreover, the difference between cell types also needs to be analyzed during the choice for the target, in order to correctly extrapolate the resuls to human health.

In this review it was found one study [Fort *et al.*, 2001] on reproduction toxicity of, the pesticide boric acid, among other chemicals. The evaluation was done exposing frogs, *Xenopus laevis*, to a sublethal concentration for a period of 30 days, by culture water (mimetizing the environmental exposure route). This study wasn't inserted on this review, because alternatives aim to reduce the use of animals in a method, or refine the use or replace the animal use for other non-animal target, even though some researchers consider an alternative method the use of invertebrates[11].

Research on replacement methods needs to be encouraged as it could lead to significantly reduce the current massive use of animals in experimental science. It is important to note that this research, as well as most attempts to identify works on alternative methods, failed to analyze studies on in vitro tests when they weren't declaredly intended for these objectives. Thus, it would be very interesting for the global scientific community that research groups on in vitro technology start to consider their methodologies to contribute on the development of novel alternatives to animal testing and, moreover, that they express this intent in their studies.

Limitations

After the selection of records, it was difficult to access of several old articles or works published in periodicals which do not meet free or sponsored access in Brazil, which considerably delayed the analysis.

Acknowledgments

The authors acknowledge the financial support from Coordination for the Improvement of Higher Level Personnel (Capes) and Research Support Foundation of Rio de Janeiro (FAPERJ). This study couldn't occur without the help and assistances of librarians of Sérgio Arouca National School of Public Health, specially to Mrs. Gizele Rocha who gently taught us to develop different strategies in many databases.

References

1. Mansour SA (2004) Pesticide exposure-Egyptian scene. Toxicology 198: 91-115.

2. Gupta PK (2004) Pesticide exposure-Indian scene. Toxicology 198: 83-90.

3. Yáñez L, Ostiz D, Calderón J, Batres L, Carrizales L, et al. (2002) Overview of Human Health and Chemical Mixtures: Problem Facing Developing Countries. Environmental Health Perspectives 110: 901-909.

4. Caldas PE, Boon, Tressou J (2006) Probabilistic assessment of the cumulative acute exposure to organophosphorus and carbamate insecticides in the Brazilian diet. Toxicology 222: 132-142.

5. CTTAEA (2007) Toxicity Testing in the 21st Century: A Vision and a Strategy.

6. Russell W, Burch R (1959) The Principles of Humane Experimental Technique; Methuen, editor. London. 238 p.

7. Rispin A, Farrar D, Margosches E, Gupta K, Stitzel K, et al. (2002) Alternative methods for the median lethal dose (LD(50)) test: the up-and-down procedure for acute oral toxicity. ILAR J 43: 233-243.

8. Diener W, Mischke U, Schlede E, Kayser D (1995) The biometrical evaluation of the OECD modified version of the acute toxic class method (oral). Arch Toxicol 69: 729-734.

9. Schlede E, Mischke U, Diener W, Kayser D (1995) The international validation study of the acute toxic class method (oral). Arch Toxicol 69: 659-670.

10. Fernandez Freire P, Peropadre A, Perez Martin JM, Herrero O, Hazen MJ (2009) An integrated cellular model to evaluate cytotoxic effects in mammalian cell lines. Toxicol In Vitro 23: 1553-1558.

11. Rajini PS, Melstrom P, Williams PL (2008) A comparative study on the relationship between various toxicological endpoints in Caenorhabditis elegans exposed to organophosphorus insecticides. J Toxicol Environ Health A 71: 1043-1050.

12. Martins JD, Monteiro JP, Antunes-Madeira MC, Jurado AS, Madeira VM (2003) Use of the microorganism Bacillus stearothermophilus as a model to evaluate toxicity of the lipophilic environmental pollutant endosulfan. Toxicol In Vitro 17: 595-601.

13. Kowalska-Pylka H, Majer-Dziedzic B, Niewiadomy A, Matysiak J (2001) Evaluation of the toxicity of substituted benzthioanilides by using in vitro tests. Altern Lab Anim 29: 547-556.

14. Bertheussen K, Yousef MI, Figenschau Y (1997) A new sensitive cell culture test for the assessment of pesticide toxicity. J Environ Sci Health B 32: 195-211.

15. Figenschau Y, Yousef MI, Sveinbjornsson B, Bertheussen K (1997) A sensitive serum-free colorimetric assay for the detection of cytotoxic effects of pesticides. J Environ Sci Health B 32: 177-194.

16. Koch HP, Hofeneder M, Bohne B (1993) The yeast test: an alternative method for the testing of acute toxicity of drug substances and environmental chemicals. Methods Find Exp Clin Pharmacol 15: 141-152.

17. Castano A, Bols N, Braunbeck T, Dierickx P, Halder M, et al. (2003) The use of fish cells in ecotoxicology. The report and recommendations of ECVAM Workshop 47. Altern Lab Anim 31: 317-351.

18. Zitova A, O'Mahony FC, Cross M, Davenport J, Papkovsky DB (2009) Toxicological profiling of chemical and environmental samples using panels of test organisms and optical oxygen respirometry. Environ Toxicol 24: 116-127.

19. Zurita JL, Jos A, Camean AM, Salguero M, Lopez-Artiguez M, et al. (2007) Ecotoxicological evaluation of sodium fluoroacetate on aquatic organisms and investigation of the effects on two fish cell lines. Chemosphere 67: 1-12.

20. Ohno Y, Miyajima A, Sunouchi M (1998) Alternative methods for mechanistic studies in toxicology. Screening of hepatotoxicity of pesticides using freshly isolated and primary cultured hepatocytes and non-liver-derived cells, SIRC cells. Toxicol Lett 102-103: 569-573.

21. Bull S, Langezaal I, Clothier R, Coecke S (2001) A Genetically engineered cell-based system for detecting metabolism-mediated toxicity. Altern Lab Anim 29: 703-716.

22. Montesissa C, De Liguoro M, Amorena M, Lucisano A, Carli S (1995) In vitro comparison of aldicarb oxidation in various food-producing animal species. Vet Hum Toxicol 37: 333-336.

23. Mancebo A, Hernandez O, Gonzalez Y, Aldana L, Carballo O (2008) Assessment of skin and eye irritation of 14 products under the stepwise approach of the OECD. Cutan Ocul Toxicol 27: 173-185.

24. Kishore AS, Surekha PA, Sekhar PV, Srinivas A, Murthy PB (2008) Hen egg chorioallantoic membrane bioassay: an in vitro alternative to draize eye irritation test for pesticide screening. Int J Toxicol 27: 449-453.

25. Tavaszi J, Budai P, Palovics A, Kismanyoki A (2008) An alternative test battery in detecting ocular irritancy of agrochemicals. Commun Agric Appl Biol Sci 73: 891-895.

26. Tavaszi J, Budai P (2006) Toxicity study of agrochemicals on chorioallantoic membrane of the egg. Commun Agric Appl Biol Sci 71: 101-105.

27. Tavaszi JB, P (2007) The use of HET-CAM test in detecting the ocular irritation. communications in agricultural and applied biological sciences 72: 137-141.

28. Budai PV, L (2000) In vitro ocular irritation toxicity study of some pesticides. ACTA VETERINARIA HUNGARICA 48: 221-228.

29. Prinsen MK (1996) The chicken enucleated eye test (CEET): a practical (pre)screen for the assessment of eye irritation/corrosion potential of test materials. Food Chem Toxicol 34: 291-296.

30. van de Sandt JJ, Meuling WJ, Elliott GR, Cnubben NH, Hakkert BC (2000) Comparative in vitro-in vivo percutaneous absorption of the pesticide propoxur. Toxicol Sci 58: 15-22.

31. Lazzari G, Tessaro I, Crotti G, Galli C, Hoffmann S, et al. (2008) Development of an in vitro test battery for assessing chemical effects on bovine germ cells under the ReProTect umbrella. Toxicol Appl Pharmacol 233: 360-370.

32. Oskarsson A, Ulleras E, Plant KE, Hinson JP, Goldfarb PS (2006) Steroidogenic gene expression in H295R cells and the human adrenal gland: adrenotoxic effects of lindane in vitro. J Appl Toxicol 26: 484-492.

33. Baron R (1994) A Carbamate Insecticide: A Case Study of Aldicarb. Environ Health Perspect 102: 5.

34. ICCVAM (2001) The Revised Up-and-Down Procedure: A Test Method for Determining the Acute Oral Toxicity of Chemicals.

35. Falk H (2003) In: ATSDR, editor. North Carolina.

36. McClellan M (2003) In: FDA, editor.

37. Merenda J (2003) In: EPA, editor.

Annexes

Table 1. Distribution of study fields

Field	Aims	Study design (n° of tudies)	Reference
Acute toxicity (LD 50)	Reduction	Statistical strategy (3)	[7,8,9]
	Reduction (screening of substances) or replacement	Yeast (1), animal cell line (5), bacterial (1)	[10,11,12,13,14,15,16]
Ecotoxicity	ECVAM report	Fish cells	[17]
	Reduction (screening of substances) or replacement	Bacterial (2), algae test (1), fish cell line (2), planktonic crustacean (1), fish embryo test (1)	[18,19]
Hepatotoxicity	Reduction (screening of substances) or replacement	Animal cell line (1), primary hepatocyte culture (1), fresh hepatocyte (1)	[20].
	Basic study on drug metabolite.	Animal cell line (1), Hepatocyte from several species (1)	[21,22].
Eye irritation	Screening of substances by HET-CAM	Chorioallantoic Membrane (6)	[23,24,25,26,27,28]
	Screening of substances by enucleated eye test	Chicken eyes	[29]
Other	Skin permeability	Rats (1), human (1), rat and human skin membranes (1),	[30]

		pig-ear model (1)	
	Neurotoxicity	Nematode (1)	[11]
	Reproductive toxicity	Bovine oocyte (1)	[31]
	Adrenotoxic - endocrine **disruptor**s	Human adrenocortical cell line (1)	[32]

LD50: Lethal dose, 50% is the dose that kills half (50%) of the animals tested and it is a standardized measure for expressing and comparing the toxicity of chemicals.

Table 2: Compilation resulted of all studies on HET-CAM test.

Product	IS (HET-CAM)	IT % (HET-CAM)	Classification (HET-CAM)	Draize Score	*In vivo* result	Reference
NeoNim 60	17.40 (0.65)	-	Severe irritant	0.87	Mildly irritant C2A	[23]
OleoNim 80	19.16 (0.60)	-	Severe irritant	1.83	Eye irritant C1	[23]
Oleoresin capsicum 20%	12.02 (0.08)	-	Severe irritant	0.13	Non-irritant	[23]
Tetraconazole 96.5%	19.19	10	Moderate	-	Moderate	[24][1]
Bronopol 80%	17.5	5	Irritant	-	Irritant	[24][1]
Mancozeb 85%	0	10	Nonirritant	-	Non-irritant	[24][1]
Fenbuconazole 97.5%	0	10	Nonirritant	-	Non-irritant	[24][1]
Paclobutrazole 95.7%	0	10	Nonirritant	-	Moderate	[24][1]
Previcur 607 SL	4.7	-	Weak irritation	14	Minimally	[27]
Substral	4.8	-	Weak irritation	3.3	Minimally	[27]
Systhane 12 E	11.5	-	Severe irritation	81	Extremely	[27]
Agrol	4.8	-	Weak irritation	15.3	Mildly	[27]
Topas	11.55	-	Severe irritation	75	Extremely irritation	[27]
Omite 57 E	10.21	-	Severe irritation	85	Extremely irritation	[27]
Folicur Solo	11.60	-	Severe irritation	36.7	Severe	[25]

Silwet L-77	11.75	-	Severe irritation	70.7	Extreme	[25]
Kohinor 200 SL	10.64	-	Severe irritation	22.50	Moderate	[25]
Falcon 460 EC	11.77	-	Severe irritation	41.7	Severe	[25]
Previcur 607 SL	4.7	-	Weak irritation	14	Minimally	[25]
Confidor 200 SL	4.92	-	Weak irritation	12.67	Minimally	[25]
Charisma 207 EC	4.02	-	Weak irritation	-	No irritation[2]	[26]
Unifosz 50 EC	4.94	-	Weak irritation	-	Irritation[2]	[26]
Confidor SL 200	4.92	-	Weak irritation	-	No irritation[2]	[26]
Fozat 480	4.94	-	Weak irritation	-	Weak irritation[2]	[26]
Sumi-Alpha 5 EC	4.96	-	Moderate irritation	-	Mild irritation[2]	[26]
Chinmetrin	5.02	-	Moderate irritation	-	Mild irritation[2]	[26]
Arelon 500 FW	5.71	-	Moderate irritation	8	Slight irritation	[28]
Banvel 480 S	11.05	-	Severe irritation	65	Severe irritation	[28]
Dikamin D	9.14	-	Severe irritation	55	Severe irritation	[28]
Karathane LC 10%[3]	5.06	-	Moderate irritation	52	Severe irritation	[28]
Ronstar	5.28	-	Moderate irritation	49	Moderate irritation	[28]
Modown 4 F 10%[3]	1.94	-	Weak irritation	4	No irritation	[28]

1. *In vivo* reported data cited in the original article.
2. *In vivo* Draize reported data cited in the original article, from Luepke and Wallat, 1987.
3. Irritation index to 100% pesticide concentration was not showed in the original article.

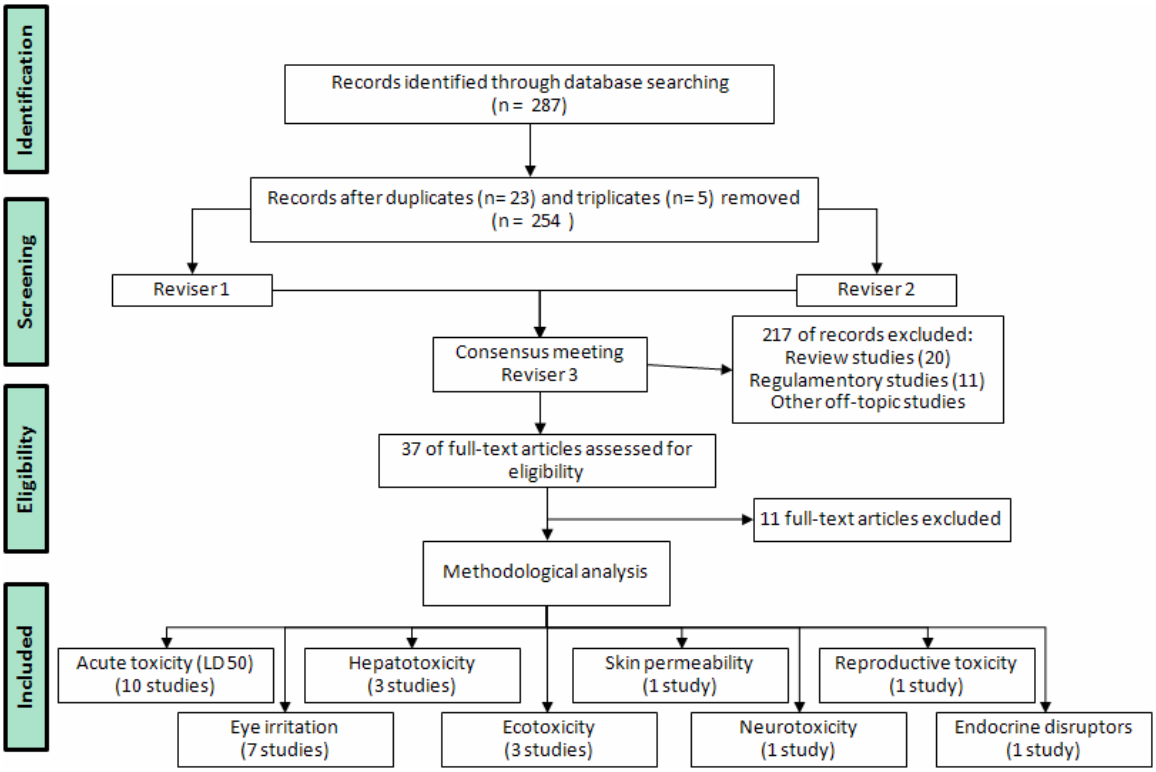

Figure 1. Flow chart of systematic literature search

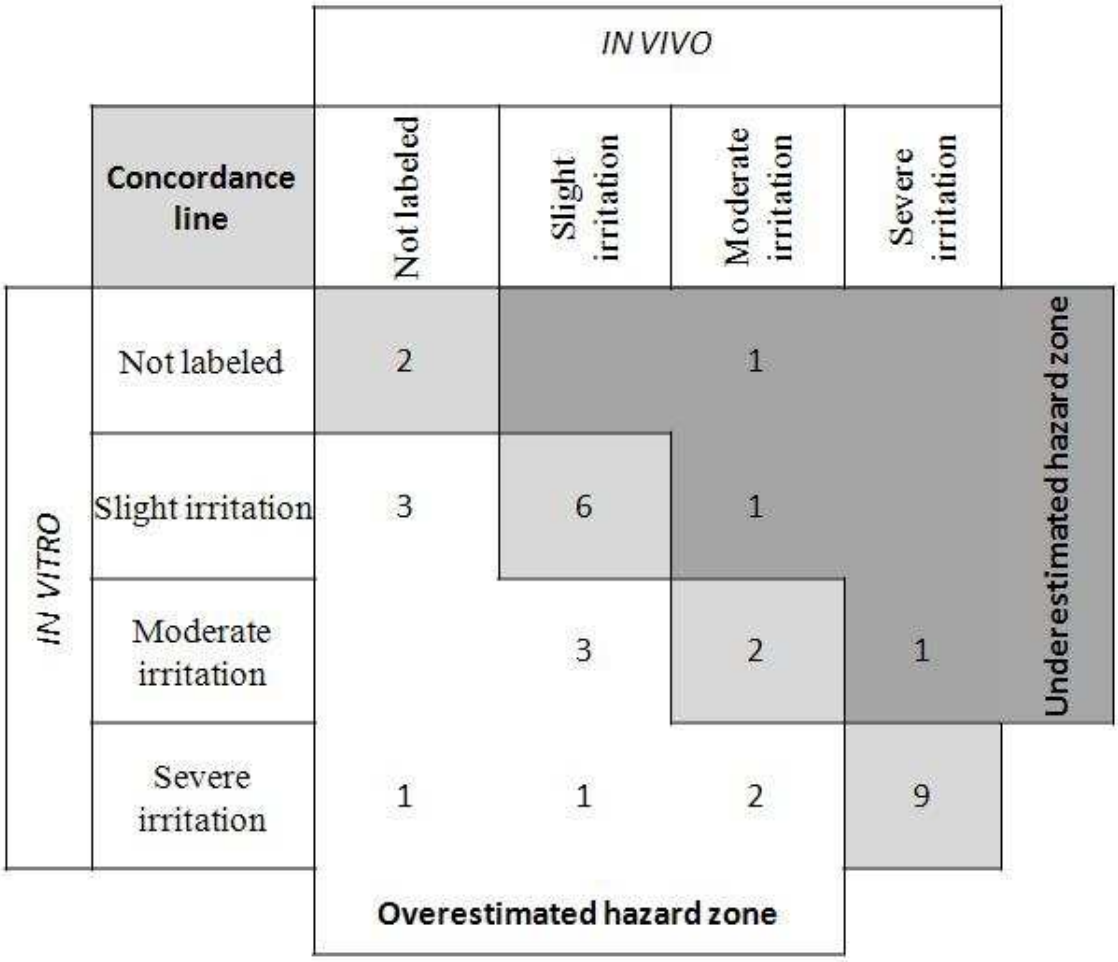

Figure 2. General comparison between in vitro and in vivo pesticide classification from the studies on eye irritation. Dark gray zone represents the results which were underestimated by *in vitro* tests, as the scores were lower than *in vivo*.

5.2. ARTIGO 2: A high-throughput test using cellular acetylcholinesterase activity without cell injury

Este espaço foi deixado em branco propositadamente.

A high-throughput test using cellular acetylcholinesterase activity without cell injury

Abstract

Acetylcholinesterase (AChE) is a membrane-bound enzyme with extracellular activity. It is an important enzyme studied in neurotoxicology and neuropathology, since it is involved in the neural system development, as well as in several other neurophysiological processes.

In the present work, a modified Ellman's method for AChE activity assay with preservation of cell integrity is proposed, allowing its use on multiparametric *in vitro* neurotoxicological test batteries.

Phosphate buffered saline (PBS) or Dulbecco's modified Eagle's medium (DMEM) high glucose, with or without 10% fetal bovine serum (FBS), added with 1 mM acetylthiocholine (AceScol) and either 0.150 mM or 0.050 mM of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were mixed on 96 wells microplate, following $O.D._{412}$ for 120 minutes. The reaction medium with the lowest background (0.012 $O.D._{412}$/h). was PBS, which was subsequently tested in the presence of different undifferentiated neuroblastoma (SH-SY5Y) cells, seeded 24 h before at different cell densities.

AChE activity was evaluated in different substrate concentrations and the presence or absence of two inhibitors: quinidine sulfate and aldicarb, a carbamate pesticide. Cell damage by reaction medium (DTNB) and products (thionitrobenzoic acid or TNB) was assessed by mitochondrial dehydrogenase activity (XTT assay).

FBS induced a fast degradation of AceScol, possibly due to the presence of butyrylcholinesterase. In the presence of cells, it was possible to detect enzyme activities using 1mM of AceScol and 0.05 mM of DTNB in PBS, allowing up to 0.6 $O.D._{412}$/h, which represents an activity of about 30µU of AChE per well seeded with $12x10^4$ cells.

Changes in the pattern of DTNB reaction on microplate wells with and without neuroblastoma cells also indicate a possible method to measure AChE activity of these cells. This new method may reduces costs, experimental time and procedures and, therefore, can contribute to future replacements of animal testing in neurotoxicology.

Introduction

Acetylcholinesterase (AChE) is an important enzyme involved in the rapid hydrolysis of the neurotransmitter acetylcholine. This neurotransmitter is protagonist in numerous cholinergic pathways in the central and peripheral nervous system, from vegetative functions (heart rate and blood pressure) to muscular contraction and cognitive function. The inhibition of AChE may have severe consequences and alters diverse functions including actions on the autonomic nervous system (i.e., salivation, lacrimation, urination, and defecation), somatic nervous system (tremors), and central nervous system (convulsions) and can lead to death from a combinations of central and peripheral effects (bronchiolar constriction, paralysis of the respiratory muscles, and so forth) [1,2].

Due to this physiological impact, cholinesterase inhibitors are widely used as pesticides in agriculture and even as weapons in chemical warfare. Organophosphate compounds were synthesized during World War II as both insecticides and nerve gases, and shortly thereafter carbamates (e. g., Aldicarb) were developed as a safer alternative to them [2]. Both insecticides, organophosphates and carbamates, form an enzyme-inhibitor complex, generating phosphorylated or carbamoylated enzymes. Phosphorylated AChE is relatively stable, so that inhibition is virtually irreversible, but carbamoylated AChE can be hydrolyzed regenerating the original form of active enzyme [3].

In Pharmacology, AChE inhibitors are used since the mid – 1800s [2] and have been explored in several different animal models. A study with monkeys demonstrated that AChE, acting on the muscarinic cholinergic neuronal transmission, is involved in memory and cognitive functions and its inhibition can enhance memory functions [4]. These data are consistent with the cholinergic deficit hypothesis of Alzheimer Disease (AD) due to the fast degradation of acetylcholine and thus allowing a treatment by administration of AChE inhibitors [5,6,7].

The use of animal models in toxicology and pharmacology has been criticized in all social fields and a new toxicological paradigm is developing, focusing on the alternatives to animal testing, based on the 3R (reduce, refine and replace) concept [8,9]. The nematode *Caenorhabditis elegans* had been indicated as a model for neurotoxicological studies, measuring movements for estimating the action of pesticides on cholinergic system [10]. As of April 2010, only one study may be found on literature pertaining alternatives to pesticide toxicology, using the nematode model [11].

The present work aims to propose a high-throughput human-enzyme method based in Ellman's method [12]. The use of living cells for AChE activity determination can enable a test of AChE inhibitors for human and environmental toxicology and for pharmacological studies of new drugs. The method can be used to analyze cell adaptations to new conditions due to chemical exposure, enabling molecular, cellular and systemic interactions studies.

Materials and Methods

Cell culture

Human neuroblastoma SH-SY5Y cell line was purchased from Rio de Janeiro Cell Bank (depositary of American Type Culture Collection- ATCC). Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) supplemented with high glucose and 10% fetal bovine serum (FBS) without antibiotics. They were kept at 37 °C in a humidified atmosphere with 5% CO_2 in air. Cells were subcultured once reached 80% confluence cell layer. The medium was changed every 3–4 days. Passages 37-42 were used in this study.

Determination of acetylthiocholine hydrolysis in possible test media

The Ellman method [12] was used to identify and quantify acetylcholine hydrolysis and thiocholine production. On 96 wells microplate without cells, 160 µL of phosphate buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM) high glucose, with or without 10% fetal bovine serum (FBS), or only FBS, were added with 1 mM acetylthiocholine (AceScol) and either 0.150 mM or 0.050 mM of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid). The $O.D._{412}$ was followed for 40 minutes on a microplate reader (Synergy II, BioTek Instruments, Winooski, VT, USA).

Determination of acetylcholinesterase activity

Phosphate buffered saline (PBS) was added with 1 mM acetylthiocholine (AceScol) and either 0.150 mM or 0.050 mM of DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) on 96 wells microplates, on a final volume of 160 uL, in wells with $4x10^4$, $8x10^4$ or $12x10^4$ cells seeded 24 h before (undifferentiated neuroblastoma), and the $O.D._{412}$ was followed for 120 minutes. The same solutions were added to wells without cells to determine non-enzymatic hydrolysis of AceScol, while the same concentrations and volume of DTNB without AceScol was added in wells with cells to determine the non-specific reactivity of DTNB with free thiol groups.

Acetylcholinesterase inhibition test

The enzyme inhibition was analyzed in the presence of either quinidine sulfate or Aldicarb, a carbamate insecticide, both with well-known anti-cholinesterase action. Cells were exposed to 5 mM of quinidine sulfate.at different cell densities (40, 80 and 120 thousand cells). The test for Aldicarb consisted on $12x10^4$ seeded cells exposed to 250, 500, 750 or 1000 uM of the pesticide by 30 minutes. After exposure, the cells were washed two times with PBS and the acetylcholinesterase activity was analyzed as described on the previous section.

XTT Assay

The XTT test is based on the ability of mitochondrial dehydrogenase enzymes to convert the yellow water-soluble tetrazolium salt (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl) -5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (In Citotox, Xenometrix) into orange-colored soluble compounds of formazan[13], measured by their absorbance at 480 nm measured with a microplate UV–vis spectrophotometer (PowerWave MS2, BioTek Instruments, Rio de Janeiro, RJ, Brazil).

Results and Discussion

Photometric analysis of TNB (thionitrobenzoate)

The absorptivity of TNB on 96- microplates was assessed by the DTNB reaction with different concentrations of L-cysteine (from 0.001 to 0.1 mM) resulting 5,726 M^{-1} at 412 nm. The Coefficient of Determination (r^2) was higher than 0.999 and the Standard Deviations were insignificant (Figure 1). The molar absorptivity of TNB for an optical path of 1cm was 14,150 $M^{-1}.cm^{-1}$, that indicates an optical path of 0.4 cm. It is compatible with wells height that was used for determining AChE activity, as International Units. SH-compounds react instantly with DTNB, as was confirmed by measures without differences from 5 to 20min.

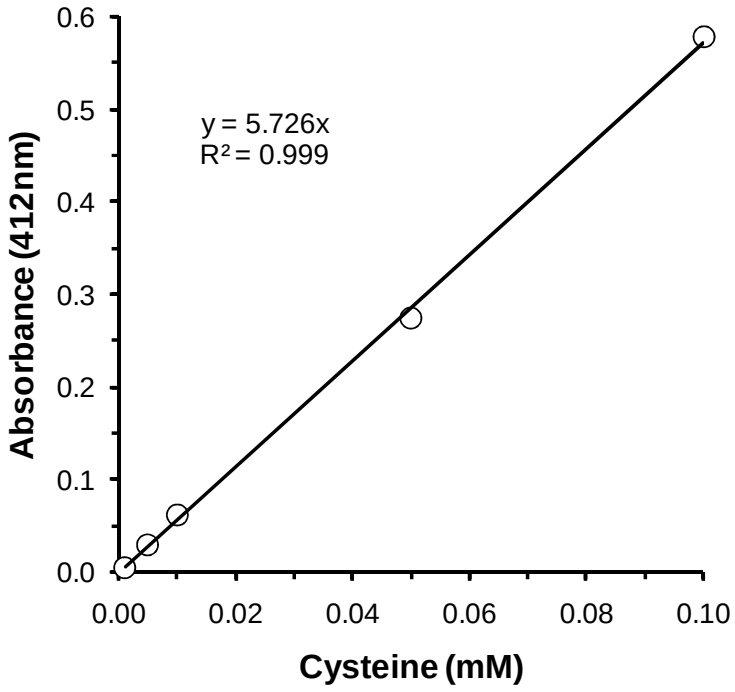

Figure 1 - Absorptivity of TNB on 96- microplate wells. Cysteine was dissolved in PBS to 160µL of final volume and the absorbance was monitored between 5 to 20min.

Selection of test medium

Fetal bovine serum presented a high background (approx. 1.0 OD_{412}) and a high reactivity of ASch in DTNB 0.05 and 0.15 mM (approx. 2.5 and 1.7 $OD_{412,}$ respectively). DMEM supplemented with 10% FBS presented the same behavior as FBS. DMEM free-serum also hydrolyzed Asch, even though at slower rates (0.126 and 0.135 $O.D._{412}$/h to 0.05 and 0.15 mM DTNB, respectively). PBS showed the lowest background (respectively approx. 0.10 and 0.11 OD_{412}). and Asch hydrolysis (0.012 and 0.014 $O.D._{412}$/h to DTNB 0.05 and 0.15 mM, respectively). We could not discard the

possible presence in FBS of soluble plasma cholinesterases, Butyrylcholinesterase (BChE), and other thiol products.

Acetylcholinesterase detection

The mean absorbances found for both concentrations of DTNB (0.05 and 0.15 mM) and with different cell densities (Table 1) demonstrated that, in order to obtain O.D. levels higher than 0.2, the incubation time should be superior to two hours. Nevertheless, the correlation between readings, which was always higher than 0.97 showed that the reaction is constant and stable enough at times inferior to 2 h.

Table 1: Optical Density ($O.D._{412}$ nm) of TNB resultant from the Ellman reaction, with different cell densities and DTNB concentrations.

Cells seeded per well	**O.D.412nm/h**	
	DTNB 0.05 mM	**DTNB 0.15 mM**
$12x10^4$	0.075 (0.998)	0.076 (0.997)
$8x10^4$	0.052 (0.996)	0.058 (0.997)
$4x10^4$	0.023 (0.974)	0.020 (0.989)

O.D.412nm/h = slope of the Linear Regression between Absorbance (412nm) and time for at least 2 hours and, inside parentheses, the Coefficient of Determination (r^2)

The variation on DTNB(Figure 2)., as the linear functions obtained with either 0.05 or 0.15mM DTNB were very similar. Therefore, it is recommendable the use of the slower concentration to prevent interactions with the cells. It is also interesting the use of cell densities between $8x10^4$ and $12x10^4$ cells, in order to obtain faster substrate degradation and a better cell concentration, making the method more parsimonious.

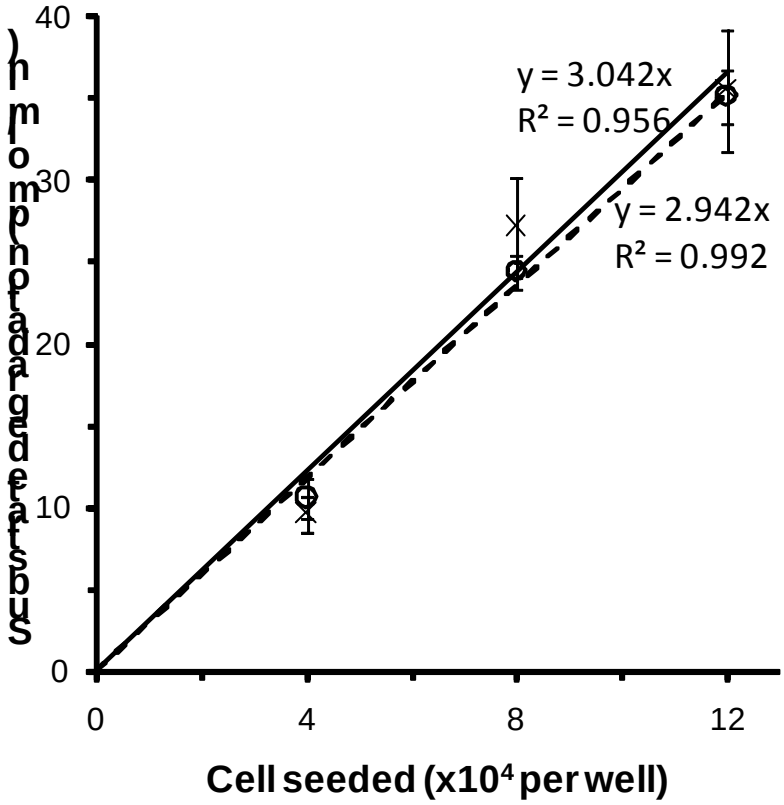

Figure 2. Variation of substrate degradation by cell and DTNB concentration. Dotted line = tendency to DTNB 0.05 mM; full line = tendency to DTNB 0.15 mM.

Acetylcholinesterase inhibition and cellular viability

Both anti-cholinesterase had a strong effect on the enzyme velocities. Figure 3 shows the effects of 5 mM quinidine sulfate, a well known inhibitor of cholinesterase activity, on substrate degradation in different cell densities. The enzyme activity had a sharp decline in every cell density tested. The test with this inhibitor had not a incubation time, possibly the time of exposure affect the inhibition, reducing the velocity yet. Quinidine sulphate has a slow affinity with AChE, so it needs a longer incubation time. Due to it had been used like a fast inhibitor of BchE in plasma test [14]. However the concentration used to this test was sufficiently high to cause a considerable inhibition of AchE.

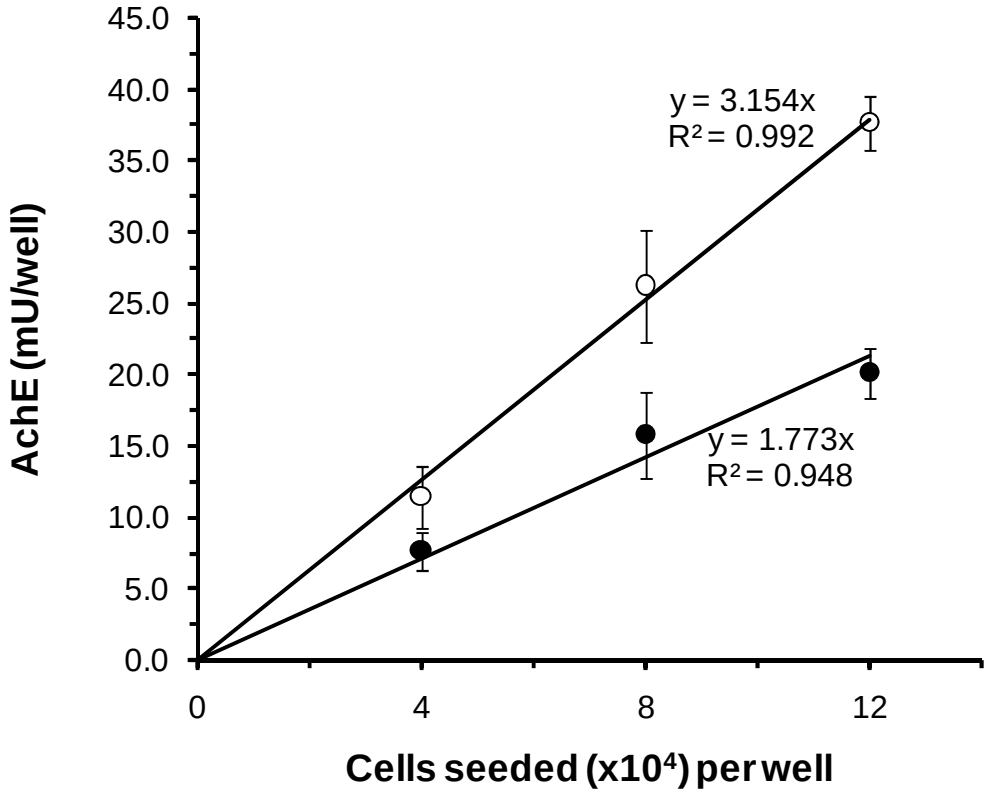

Figure 3. Influence of quinidine sulfate in the AchE activity. (black circles = with 5 mM quinidine sulfate; empty circles = control without inhibitor)

Once it has been demonstrated that the methodology was able to detect the effects of a strong inhibitor of AChE, it was performed a test to verify if it would be also sensible enough to detect the anti-cholinesterase action of Aldicarb, a widely employed cabamate insecticide. Indeed, AChE inhibition was demonstrated to Aldicarb after 30 minutes of incubation with cultured cells (Figure 4). The Aldicarb concentrations had a higher effect on the AChE inhibition than cellular viability, demonstrate by the concentration to cellular inhibition 50% was higher than the concentration to enzyme inhibition 50% (1.962 mM and 0.608 mM respectively). These Aldicarb concentrations were high and it would be expected to found inhibition in lower concentrations, because the test occurred on little quantities of enzymes. But the carbamoylated enzymes formed by Aldicarb are really instable and the process to analyze the AchE activity possibly regenerated much of these inhibited forms to enzyme's active form. Nevertheless, this experiment demonstrated an influence of an AChE inhibitor on the substrate degradation besides the cellular effects.

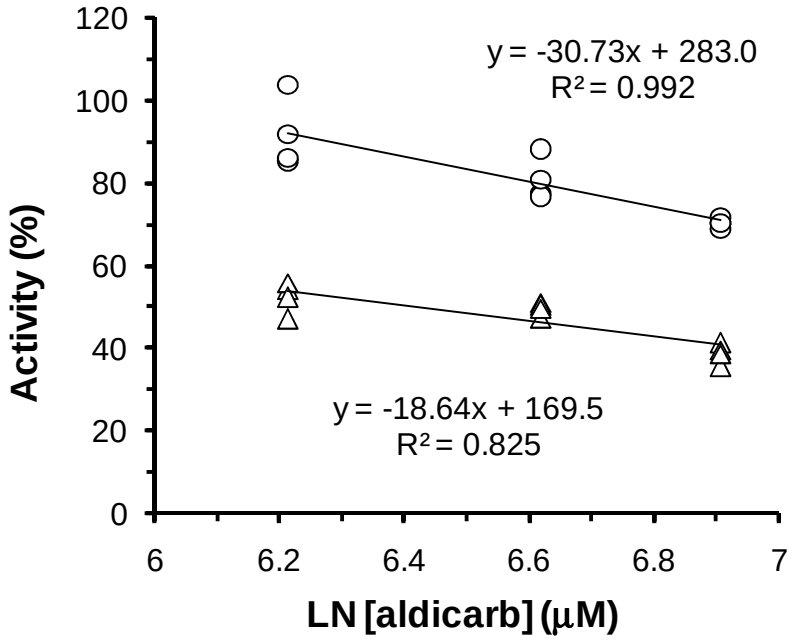

Figure 4. AChE and Cellular inhibition by the carbamate Aldicarb. Triangle: AChE activity; Circle: Cell viability by mitochondrial enzyme activity (XTT)

It may be considered that batteries of tests are necessary to obtain more complete toxicological and pharmacological assessments of chemical substances. These batteries may include tests on the chemical action on other targets, as well as the metabolization of such substances. Aldicarb, for example, is rapidly metabolized to aldicarb sulfoxide (ASX) and aldicarb sulfone (ASN). ASX can decrease the AChE inhibition, while the conversion to ASX is considered a process of bioactivation. Therefore, models of interactions between different cell lines and process should be considerate before of the extrapolation to complex organisms. The most striking feature of the methodology discussed in the present work resides on the cell survival after the AChE assessment, allowing for further in vitro studies on the same exposed cells.

This work intends to guide new studies to use of in vitro cell models for the assessment of AChE and its alterations after exposure to chemicals of commercial, environmental or health relevance. Cell culture models can contribute to the development of new alternative methods to neurotoxicology and neuropharmacology based on AChE proprieties and the evaluation of cytotoxicity and cytocompatibility on the same culture. In this work it was used only undifferentiated neuroblastoma cells, speeding the analyzes. Whereas only one day is necessary to test on undifferentiated cells, the process of cell differentiation spends 6 days. However, the same method should be standardized to other cell lines, and further studies on expression of intracellular AChE molecules and degradation of this enzyme [15], should be performed sequentially, bringing a

more complete overview on the potential use of this enzyme as a marker for toxicity in preclinical assays. Thereafter a validation study, using differentiate and undifferentiated cells, also should be considerate to different objectives.

References

1. Chambers J, Oppenheimer S (2004) Organophosphates, Serine Esterase Inhibition, and Modeling of Organophosphate Toxicity. Toxicol Sci 77: 185-187.

2. Moser V (1995) Comparisons of the acute effects of cholinesterase inhibitors using a neurobehavioral screening battery in rats. Neurotoxicol Teratol 17: 617-625.

3. Baron R (1994) A Carbamate Insecticide: A Case Study of Aldicarb. Environ Health Perspect 102: 5.

4. Tsukada H, Nishiyama S, Fukumoto D, Ohba H, Sato K, et al. (2004) Effects of acute acetylcholinesterase inhibition on the cerebral cholinergic neuronal system and cognitive function: functional imaging of the conscious monkey brain using animal PET in combination with microdialysis. Synapse 52: 1-10.

5. Benzi G, Moretti A (1998) Is there a rationale for the use of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease? Eur J Pharmacol 346: 1-13.

6. Polinsky R (1998) Clinical pharmacology of rivastigmine: a new-generation acetylcholinesterase inhibitor for the treatment of Alzheimer's disease. Clinical therapeutics 20: 634-647.

7. Bores G, Huger F, Petko W, Mutlib A, Camacho F, et al. (1996) Pharmacological evaluation of novel Alzheimer's disease therapeutics: Acetylcholinesterase inhibitors related to galanthamine. J Pharmacol Exp Ther 277: 728-738.

8. (CTTAEA) CoTTaAoEA (2007) Toxicity Testing in the 21st Century: A Vision and a Strategy.

9. Russell W, Burch R (1959) The Principles of Humane Experimental Technique; Methuen, editor. London. 238 p.

10. Wren J, Kille P, Spurgeon D, Swain S, Sturzenbaum S, et al. (2011) Application of physiologically based modelling and transcriptomics to probe the systems toxicology of aldicarb for Caenorhabditis elegans (Maupas 1900). Ecotoxicology 20: 397-408.

11. Rajini PS, Melstrom P, Williams PL (2008) A comparative study on the relationship between various toxicological endpoints in Caenorhabditis elegans exposed to organophosphorus insecticides. J Toxicol Environ Health A 71: 1043-1050.

12. Ellman G, Courtney K, Andres Jr V, Featherstone R (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7: 88-90.

13. Scudiero D, Shoemaker R, Paull K, Monks A, Tierney S, et al. (1988) Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. Cancer Res 48: 4827.

14. Lewis P, Lowing R, Gompertz D (1981) Automated discrete kinetic method for erythrocyte acetylcholinesterase and plasma cholinesterase. Clinical Chemistry 27: 926-929.

15. Massoulié J (2008) Old and new questions about cholinesterases. Chem Biol Interact 175: 30-44.

6 – CONSIDERAÇÕES FINAIS

Na revisão sistemática foram encontrados poucos artigos que analizam a toxicidade dos agrotóxicos ou visam contribuir com novas metodologias para a redução, refinamento ou substituição do modelo animal. Provavelmente existem diversas outras pesquisas que se aplicam a esse proprósito, porém os autores não citam esse objetivo, não atingindo o público interessado nesse campo. As áreas onde se concentram o desenvolvimento de alternativas são justamente as áreas que também são bastante desenvolvidas em outros campos da toxicologia, a saber, a toxicologia de cosméticos, que são a toxicidade aguda e a irritação ocular. As mesmas metodologias utilizadas a esta área foram aplicadas aos pesticidas. Este trabalho foi importante como divulgação das pesquisas que estão ocorrendo nessa área e também por buscar pesquisas antigas que não mais foram trabalhadas, porém que possuem grande potencial de desenvolvimento.

Por ser a AChE uma enzima de membrana extremamente eficiente e com o seu sítio catalítico voltado para o lado externo, conseguimos demonstrar que é possível mensurar a sua atividade em células vivas (na linhagem de neuroblastoma humano, SH-SY5Y) através da reação da hidrólise da acetil-tiocolina e mensuração através da redução do DTNB. Essa metodologia, embora ainda necessite de mais estudos e de uma padronização, é muito importante não apenas para a toxicologia de agrotóxicos, mas também na fase de identificação de outros compostos com objetivos farmacêuticos, como drogas para Alzheimer. O uso de células possibilita também identificar produtos que apenas inibam a AChE e não causem outros danos celulares, como não foi o caso do carbamato testado, Aldicarb. A busca de bons controles e de métodos de padronização do número de células para essa metodologia devem ser investigados.

7 – REFERÊNCIAS

AMERICAN TYPE CULTURE COLLECTION (ATCC), 2011. **Product description: CRL-2266**. Disponível: www.atcc.org

ANVISA – AGÊNCIA NACIONAL DE VIGILÂNCIA SANITÁRIA. **Anvisa publica. 2009**. Disponível em: <www. anvisa.gov.br/DIVULGA/NOTICIAS/2009/ 100309_1.htm>. Acesso em: julh0 de 2011.

BALLS, M., BOTHAM, P.A., BRUNER, L.H., SPIELMANN, H. 1995. **The EC/HO international validation study on alternatives to the Draize eye irritation test.** Toxicology in Vitro 9 (6), pp. 871-929.

BARON, R. 1994. **A Carbamate Insecticide: A Case Study of Aldicarb**. Environ Health Perspect 102: 5.

BENTHAM, J. 2007. **A Utilitarian View.** *In:* KUHSE, H. & SINGER, P. (Ed.). **Bioethics: An Anthology.** Blackwell Publishing. United Kingdom. Pp. 566-567.

BIEDLER, JL; HELSON, L; SPENGLER, BA. 1973. **Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture.** Cancer research. 33. pp. 2643-2652.

BIEDLER, JL; ROFFLER-TARLOV, S; SCHACHNER, M; FREEDMAN, LS. 1978. **Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones.** Cancer research. 38. pp. 3751-3757.

BOTTINI, A. A., HARTUNG, T. **Food for thought... on the Economics of Animal Testing.** Alternatives to Animal Experimentation. 2009. 26.

BRASIL, 1989. **Dispõe sobre a pesquisa, a experimentação, a produção, a embalagem e rotulagem, o transporte, o armazenamento, a comercialização, a propaganda comercial, a utilização, a importação, a exportação, o destino final dos resíduos e embalagens, o registro, a classificação, o controle, a inspeção e a fiscalização de agrotóxicos, seus componentes e afins, e dá outras providências**. Diário Oficial da União [da República Federativa do Brasil]. 12.7.1989 Disponível em: http://www.planalto.gov.br/ccivil_03/leis/l7802.htm Acessado em: Julho, 2011.

COMBES R, GRINDON C, CRONIN MT, ROBERTS DW, GARROD JF. **Integrated decision-tree testing strategies for acute systemic toxicity and toxicokinetics with respect to the requirements of the EU REACH legislation.** Altern Lab Anim. 2008 Feb;36(1):45-63

COMMITTEE ON TOXICITY TESTING AND ASSESSMENT OF ENVIRONMENTAL AGENTS (CTTAEA), NATIONAL RESEARCH COUNCIL. **Toxicity Testing in the 21st Century: A Vision and a Strategy.** 2007.

ENCINAS M, IGLESIAS M, LIU Y, WANG H, MUHAISEN A, CEÑA V, GALLEGO C, COMELLA J X. **Sequential Treatment of SH-SY5Y Cells with Retinoic Acid and Brain-Derived Neurotrophic Factor Gives Rise to Fully Differentiated, Neurotrophic Factor-Dependent, Human Neuron-Like Cells.** Journal of neurochemistry. 75 (3). 2000

FLORES, AV; RIBEIRO, JN; NEVES, AA; QUEIROZ, ELR. 2004. **Organoclorados: Um Problema De Saúde Pública.** Ambiente & Sociedade. 7 (2): 115-15

GARTLON, J; KINSNER, A.; BAL-PRICE, A.; COECKE, S.; CLOTHIER, R.H. 2006. **Evalution of a proposed *in vitro* test strategy using neuronal and non-neuronal cell systems for detecting neurotoxicity.** Toxicology *in vitro.* 20:8. pp. 1569-1581.

JOCHEMS, CEA. 1997. **Trade and harvest of livestock sera.** Wageningen Agricultural University. Department of Animal Science Animal Husbandry/Health and Reproduction Group Utrecht University. Department of Laboratory Animal Science. Tese. Disponível: www.vet.uu.nl

JOHNSON, G; MOORE, SW. 2000. **Cholinesterases modulate cell adhesion in human neuroblastoma cells in vitro.** International Journal of Developmental Neuroscience. 18. pp. 781-790.

KOHILA, T. **Effects of Aluminum and Lead on the Rodent Cerebral Synaptosomal Integral Proteins *In Vivo* and *In Vitro.*** Academic dissertation. University of Helsinki & University of Tampere, Finland. 2004

LOPES, FM; SCHRÖDER, R; FROTA JÚNIOR, MLC; ZANOTTO-FILHO, A; MÜLLER, CB; PIRES, AS; MEURER, RT; COLPO, GD; GELAIN, DP; KAPCZINSKI, F; MOREIRA, JCF; FERNANDES, MC; KLAMT, F. 2010. **Comparison between proliferative and neuron-like SH-SY5Y cells as an *in vitro* model for Parkinson disease studies.** Brain research. 1337. pp. 85-94.

LUCHTMAN, DW; SONG, C. 2010. **Why SH-SY5Y cells should be differentiated.** Neurotoxicology. 31. pp. 164-166.

MEYER O. **Testing and assessment strategies, including alternative and new approaches.** Toxicol Lett. 2003 Apr 11;140-141:21-30

MORAGAS, WM; SCHNEIDER, MO. (2003). **Biocidas: suas propriedades e seu histórico no Brasil**. Caminhos de Geografia. 3(10): 26-40

MOSER, V. 1995 **Comparisons of the acute effects of cholinesterase inhibitors using a neurobehavioral screening battery in rats**. Neurotoxicol Teratol 17: 617-625.

MOSMANN, T. 1983. **Rapid colorimetric assay for cellular growth and survival. Application to proliferation and cytotoxicity assays**. Journal of Immunological Methods, 65. Pp. 55-63.

PERES, F; MOREIRA, JC; CLAUDIO, L.(2007) **Os impactos dos agrotóxicos sobre a saúde e o ambiente**. Ciência e saúde coletiva. 21(001): 4-5

RADIO & MUNDY. **Developmental neurotoxicity testing in vitro: Models for assessing chemical effects on neurite outgrowth.** NeuroToxicology 29 (2008) 361–376.

SANTOS, MAT; AREAS, MA; REYES. (2007) **Piretróides – Uma Visão Geral.** Alim. Nutr. 18 (3). 339-349.

SCHUMUCK, G. & AHR H. J. 1997. **Improved In Vitro method for screening Organophosphate-induced Delayed Polyneuropathy.** Toxicology In Vitro. 11. 263-270

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2009a. **Tabela 1: Casos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstâncias Registrado em 2005**. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2009b. **Tabela 6: Óbitos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstância Registrado em 2005.** Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2009c. **Tabela 1: Casos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstâncias Registrado em 2006**. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2009d. **Tabela 6: Óbitos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstância Registrado em 2006**. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2009e. **Tabela 1: Casos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstâncias Registrado em 2007**. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2009f. **Tabela 6: Óbitos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstância Registrado em 2007.** Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2010a. **Tabela 1: Casos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstâncias Registrado em 2008**. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2010b. **Tabela 6: Óbitos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstância Registrado em 2008**. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2011a. **Tabela 1: Casos de Intoxicação por Agrotóxicos de Uso Agrícola**

por Unidade Federada, Segundo Circunstâncias Registrado em 2009. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINETOX – SISTEMA NACIONAL DE INFORMAÇÕES TÓXICO-FARMACOLÓGICAS. 2011b. **Tabela 6: Óbitos de Intoxicação por Agrotóxicos de Uso Agrícola por Unidade Federada, Segundo Circunstância Registrado em 2009**. Disponível em: www.fiocruz.br/sinitox_novo/cgi/cgilua.exe/sys/start.htm?sid=123. Acessado em:julho de 2011

SINGER, P. 2000. **Ética prática.** Martins Fontes. São Paulo.

SINGER, P. 2007. **All animals are aqual.** *In:* KUHSE, H. & SINGER, P. (Ed.). **Bioethics: An Anthology.** Blackwell Publishing. United Kingdom. Pp. 568-577.

SJÖGREN B, IREGREN A, ELINDER CG, YOKEL RA. 2007. **Aluminum.** ***IN:*** NORDBERG GF, FOWLER BA, NORDBERG M, FRIBERG LT. (Org.) **Handbook on the Toxicology of Metals.** 3º ed. Academic Press, Elsevier. USA.

SKERFVING S, BERGDAHL IA. 2007. **Lead.** ***IN:*** NORDBERG GF, FOWLER BA, NORDBERG M, FRIBERG LT. (Org.) **Handbook on the Toxicology of Metals.** 3º ed. Academic Press, Elsevier. USA.

SLOTKIN, T. A. & SEIDLER, F. J. **Benzo[a]pyrene impairs neurodifferentiation in PC12 cells.** Brain Research Bulletin 80 (2009) 17–21

SOBREIRA, AE; ADISSI, PJ. 2003. **Agrotóxicos: falsas premissas e debates**. Ciênc. saúde coletiva. 8 (4)

SONG et al. **Modeling the Developmental Neurotoxicity of Chlorpyrifos in Vitro: Macromolecule Synthesis in PC12 Cells.** Toxicology and applied pharmacology 151, 182–191 (1998)

TANNE, JH. 2010. **President Obama apologises to Guatemala over 1940s syphilis study**. British Medical Journal. Disponível em: < http://www.bmj.com/content/341/bmj.c5494.full> Publicado em: 04/10/2010.

U.S. GOVERNMENT PRINTING OFFICE. 1949 **Trials of War Criminals before the Nuremberg Military Tribunals under Control Council Law** No. 10, Vol. 2, pp. 181-182. Washington, D.C.

U.S.EPA. 1997. **Guidance on Cumulative Risk Assessment, Part 1 Planning and Scoping.** Washngton, DC:U.S. Environmental Protection Agency, Science Policy Council.

VED HS, GUSTOW E, PIERINGER RA. 1991. **Regulation of neuronal differentiation in neuron-enriched primary cultures from embryonic rat cerebra by platelet activating factor and the structurally related glycerol ether lipid, dodecylglycerol.** J Neurosci Res; 30(2):353-358.

VERSTRAETEN SV, AIMO L, OTEIZA PI. **Aluminium and Lead: molecular mecanisms of brain toxicity**. Arch Toxicol (2008) 82:789–802

XENOMETRIX, 2011. **Kits in Citotox – avaliação da citotoxicidade *in vitro*.** Disponível em: /www.graulab.com

YAMAZAKI M, CHIBA K, MOHRI T. **Fundamental role of nitric oxide in neuritogenesis of PC12h cells.** British Journal of Pharmacology (2005) 146, 662–669.