**Presentation 3 - Mohamed Abou-Donia**

# Toxicological Studies Evaluating Synergism between Gulf-War Exposures

Mohamed B. Abou-Donia
Duke University Medical Center
Durham, North Carolina
donia@duke.edu

## Introduction

**Many Persian Gulf War Veterans complained of symptoms including:**

chronic fatigue,
muscle and joint pain,
ataxia,
inability to concentrate,
forgetfulness, and
behavioral abnormalities.

## Exposures

1. Between the invasion of Kuwait by Iraq on August 2, 1990 and March 1991, the U.S. Had 697,000 military personnel in the Persian Gulf Region (IOM, 1995).
2. Women accounted for 7% and the Reserve/National Guard were 17%.
3. Exposure was to a combination of psychological, biological and chemical environments.

## Hypothesis

Gulf War illnesses were caused by combined exposure to:

Pyridostigmine bromide (PB)
DEET, and
Permethrin

## Combined Chemical Exposure

**This hypothesis was prompted by**

1. Failure to identify bacterial, viral, or parasitic as a source of veterans' complaints.

2. Our previous studies that exposure to multiple chemicals increased toxicity of single compounds,

## Concurrent Chemical Exposure Increases Neurotoxicity of single Compounds

1. Methyl *iso*-butyl ketone (Non-neurotoxic) increases the neurotoxicity of the weak neurotoxicant, *n*-hexane.[a]
2. Methyl *iso*-butyl ketone Increases OPIDN induced by the OP insecticide EPN.[b]
3. The OP insecticide, Safrotin increases OPIDN induced by the OP, chlorpyrifos.[c]

[a] Biochem. Pharmacol. 41:877-883 (1991)
[b] J. Pharmacol. Exp. Therap. 257:282-289 (1991)
[c] Toxicologist 15:,205 (1995)

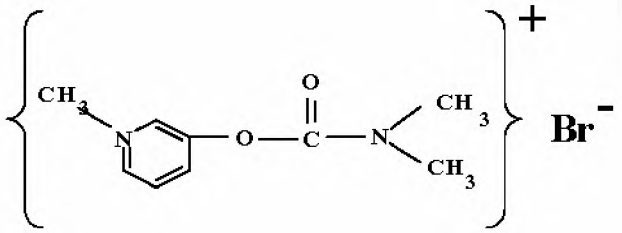
## Mechanisms of Synergism

1. **Pharmacokinetics**
   Increased neurotoxicity results from increased "effective concentration" of the neurotoxic chemical at the neurotoxicity target
   a. Activation
   b. Increased Bioavalability
2. **Pharmacodynamics**
   Alterations of the neurotoxic target, e.g., receptor up- or down-regulation

## Neurotoxicity of Pyridostigmine Bromide (PB), DEET, and Permethrin

1. All U.S. Military personnel were given PB to protect against the nerve agent, sarin.
2. Military personnel were given the insect repellent, DEET, 70% in ethanol.
3. Many military personnel used uniforms impregnated with the insecticide permethrin.

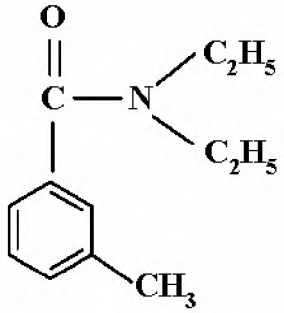
## Pyridostigmine Bromide



1. A quaternary dimethyl carbamate
2. Does not cross the BBB
3. Reversibly shields peripheral ChE (30 – 40 % inhibition)

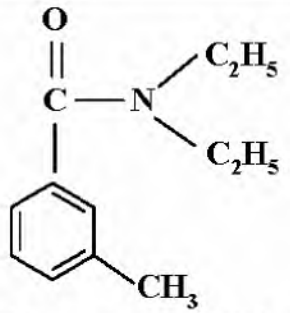
Dosage: 30 mg, 3times/day

## DEET



1. A personal insect repellant (1946).
2. Used by 30% of the population.
3. Prolonged use causes brain neuronal degeneration.

## DEET




1. **A personal insect repellant (1946).**
2. **Used by 30% of the population.**
3. **Prolonged use causes brain neuronal degeneration.**

## DEET

1. Developed by U.S. Army 1946; Registered for public use 1957; Every year used by 1/3 of US Population (75 million)

2. DEET products range from 10% to 100%, as liquids, gels, sticks, impregnated materials; more than 30 million packages are sold annually

3. Approximately 230 products containing DEET are registered with U.S. EPA by about 70 different companies

## Neurotoxicity of PB, DEET, and/or Permethrin in Hens

1. Exposure to a large dose of a single compound resulted in minimal toxicity.
2. Combination of two compounds produced greater neurotoxicity than by individual chemicals.
3. Neurotoxicity was further enhanced after concurrent administration of the three compounds

J. Toxicol. Envvron. Health 48:35-56, (1996)

## Neurotoxicity of PB, DEET, and/or Chlorpyrifos in Hens

1. Co-exposure to binary compounds caused greater neurotoxicity than single treatments.
2. Concurrent administration of the three compounds further increased neurotoxicity
3. This was indicated by: AChE activity, NTE activity, Neurological dysfunction, histopathological alterations

Fund. Appl. Toxicol. 34:201-222, (1996)

## Locomotor and Sensorimotor Performance Deficit in Rats exposed to PB, DEET, and/or Permethrin in Rats

Male, Sprague-Dawley rats were treated:

1. **Control: 70% ethanol dermal, water oral, 1 ml/kg.**
2. **PB: 1.39 mg/kg in water/d,Oral,15 days.**
3. **DEET: 40 mg/kg/d dermal in 70% ethanol, 45 days.**
4. **Permethrin: 0.13 mg/kg/d dermal in 70% ethanol, 45 days.**
5. **DEET + Permethrin, 45 days.**
6. **DEET, 45 days + PB, last 15 days.**
7. **Permethrin 45 days + PB, last 15 days.**
8. **DEET, 45 days + Permethrin 45 days + PB, last 15 days.**

Toxicol Sci. 60:305-314 (2001)

## Sensorimotor Performance

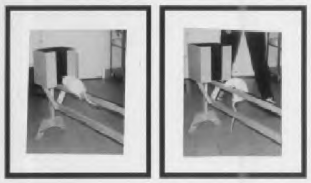
**A battery of behavioral test included:**

*a. Beam-Walk Score and Beam-Walk Time*

*b. Incline Plane Performance*

*c. Forepaw Grip*

## Beam Walk



The apparatus consists of an elevated wooden beam, a goal box with an opening located at the end of the beam, and a light source.

***BW Time:*** The time until the animal's nose entered the box (up to 90 sec.).

***BW Score:*** A 7-point scoring system for the the use of the hind paw to aid locomotion.

## Beam-Walk Time and Beam-Walk Score

***At 45 days:***

1. PB alone or in combination with DEET and/or permethrin caused significant deficit in beam-walk time and beam-walk score.
2. DEET and or permethrin did not have significant effect on beam walk-time or beam-walk score

## Incline Plane

*Description*

Rats are placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised.

The angle at which the rat begins to slip is recorded.

## Incline Plane

All Chemicals, alone or in combination, resulting in significant impairment in incline plane testing.

## Forepaw GRIP TIME

**PURPOSE:** To assess forepaw grip strength

**PROCEDURE:**

1. Have the rats grip a 5-mm diameter wood dowel
2. Time to release grip is recorded in seconds.

## Forepaw Grip Time

All Chemicals, alone or in combination, resulting in significant impairment in forepaw grip time testing.

## Sensorimotor Performance: Dose-Response

**Dose (x, mg/kg/day)**

PB,1.39, oral in water;

DEET, 40 in 70% ethanol

Permethrin, 0.13 in 70% ethanol

**Single-compound treatments**

PB, DEET, or permethrin: 0.1 x, 1 x and 10 x

**Two-compound treatments**

PB + DEET, PB + Permethrin, or DEET + Permethrin: 0.1 x, 1 x and 10 x

**Three-compound treatments**

PB + DEET + Permethrin: 0.1 x, 1 x and 10 x

J. Toxicol. Environ. Health 62:523-541 (2001)
Pharmacol. Biochem. Behav. 77:253-262 (2004)

## Plasma BChE Brain AChE, and Brain AChR: Dose-Response

1. Plasma BChE:
   PB alone slightly inhibited plasma BChE, PB in combination with DEET and/or permethrin increased its activity.
2. PB alone or in combination increased brainstem AChE activity. This leads to decreased ACh and slow down of cholinergic functions,e.g., memory deficit
3. PB alone or in combination increased ligand binding to cortex m2AChR and nAChR.

## Sensorimotor Deficit: Summary

1. Exposure to PB, DEET and permethrin, alone and in combination, causes significant sensorimotor deficits.
2. Sensorimotor deficit is associated with cortical injury.
3. Beam-walk performance involves consciousness, memory, sensorimotor, and cortical functions. An injury to the cortex is reflected by a deficit in beam-walk task.

## Brain Neuronal Cell Death Caused by DEET and/or Permethrin

**Experimental**

1. Adult, male, Sprague-Dawley rats were treated with a daily dermal dose, for 60 days with:
DEET, 40 mg/kg and Permethrin, 0.13 mg/kg

2. Twenty four hours after last dose, the animals were anesthetized and perfused via the heart with saline followed by 4% paraformaldehyde and 0.1% gluteraldehyde in Tris buffer.

## Neuropathological Studies

**The following brain areas were altered:**

1. **Motor cerebral cortex**
2. **Hippocampal formation**
   a) **CA1 subfields**
   b) **CA 3 subfields**
   c) **Dentate Gyrus**
3. **Cerebellum**

**Histopathological Assessment: Hematoxylin and eosin stain**

**Immunohistochemical Studies: Monoclonal antibodies (SMI 52) against MAP-2 and polyclonal antibodies against GFAP**

## TYPES OF NEURONS IN CEREBRAL COTRTEX

I. Molecular layer
II. External granular layer
III. External Pyramidal layer
IV. Internal granular layer
V. Internal Pyramidal layer
VI. Multiform

## Alterations in the cerebral cortex

1. Density of dying neurons was greater in deeper layer (V) and in larger pyramidal neurons of the motor cortex layer

2. Axons of these neurons form the corticospinal descending (motor) tracts, controlling the movement of muscles

3. Significant death of these neurons results in muscular weakness and loss of strength

## Neuronal degeneration of the Hippocampus

1. Hippocampus is involved in learning, memory, and emotional expression.

2. A loss of significant amount of neurons in different subfields may lead to a progressive loss of memory and results in learning disabilities.

## NEURONAL DEGENERATION OF THE CEREBELLUM

Widespread of Purkinje cell death was the hallmark lesion in the cerebellum. Since cerebellar cortex modulates cortical motor commands, its lesions may cause:

1. Delays in initiating and terminating movements.
2. Terminal tremor at the end of movement.
3. Disorders in the spatial coordination of hand and finger muscle.

## CONCLUSIONS

**Daily dermal dose of 40 mg/kg DEET and/or 0.13 mg/kg permethrin for 60 days in rats:**

1. **No change in body weight or clinical condition.**
2. **Impairment of sensorimotor performance .**
3. **Neuronal cell death in:cerebral cortex, hippocampal formation, and cerebellum.**
4. **Consequences: *Motor deficits; learning and memory dysfunction.***

## Mechanisms of Neuronal Cell Death

The results demonstrate that although DEET, an insect repellent and permethrin, an insecticide, are chemically unrelated, with different biological actions, they both produced similar histopathological lesions, both in morphology and distribution.

**Conclusion**: Both compounds have a common mechanism pathway leading to neuropathological lesions.

## Susceptibility of the Brain to Free Radical-Mediated Injury

**Free Radical-Induced Injury**

1. **Brain is rich in polyunsaturated fatty acids .**
2. **Some brain regions, e.g., substantia nigra and straiatum, have high concentration of iron.**
3. **Mitochondrial respiratory activity is higher in brain tissue, that may risk free radical "leak" from mitochondria.**

**The result is increased susceptibility of brain cell membrane damage and to lipid peroxidation.**

## NEURONAL VULNERABILITY TO ROS

***Neurons that are selectively vulnerable to reactive oxygen species (ROS) include:***

1. Cortical pyramidal neurons
2. Hippocampal CA1 pyranidal neurons
3. Cerebral Purkinje cells
4. Subpopulations in amygdala, striatum, thalamus and brainstem nuclei

## Oxidation Reaction

1. **Oxidation of small molecules by CYP450 enzymes , requires activation of molecular oxygen ($O_2$) to atomic oxygen ($O^{\bullet}$).**
2. **Most oxidative enzymes bind a metal ion, e.g., Fe, Cu, Co, or Se, which destabilizes the $O_2$ molecule .**

## Oxygen Free Radicals

A. Oxygen free radicals are intermediates in many biological reactions, but may damage macromolecules during oxidative stress.

B. Free radicals are molecules that posses a single unpaired electron in outer electron orbital, such as:

1. **Hydrogen atom, $H^{\bullet}$**
2. **Oxygen molecule, $O_2$; possesses 2 unpaired electrons**
3. **$NO^{\bullet}$**
4. **Superoxide, $O_2^{\bullet -}$ ; one unpaired electron**
5. **Hydroxyl radical, $^{\bullet}OH$**
6. **Transition metals, e.g., Cu and Fe**

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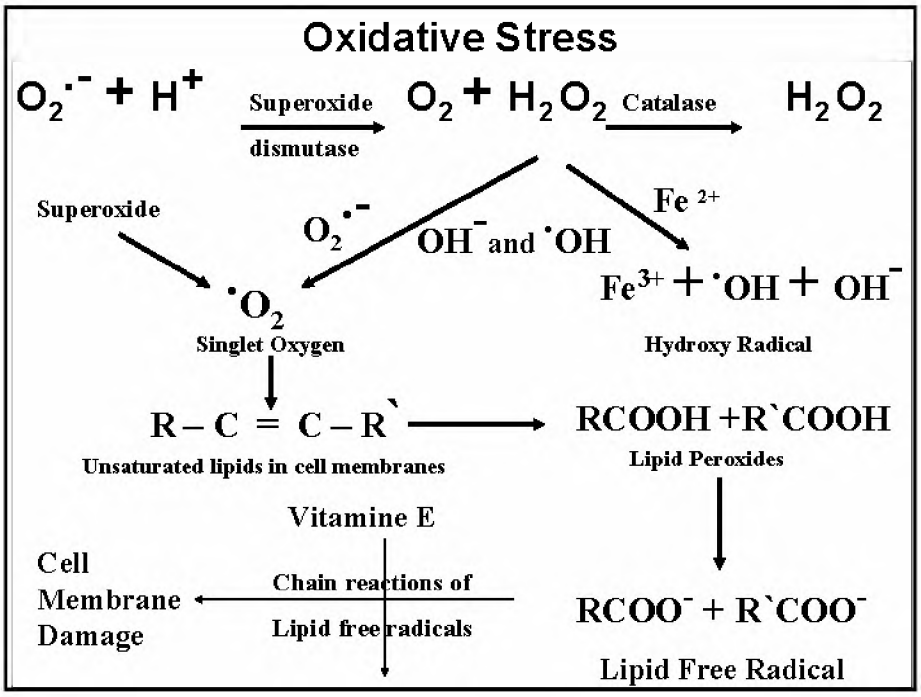
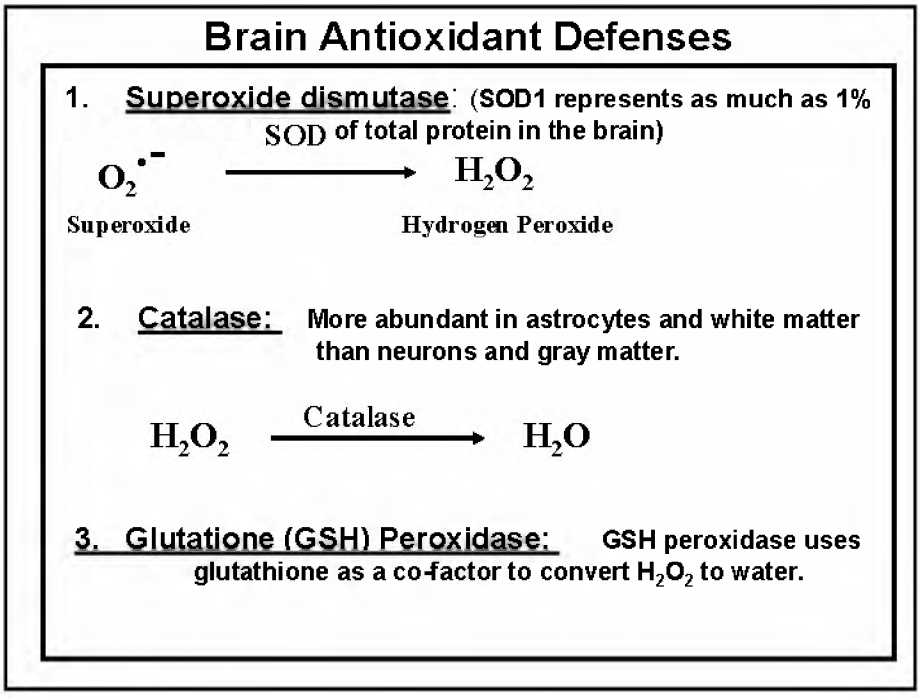

Oxidative Stress
$O_2^{\cdot -} + H^+$
Superoxide dismutase
$O_2 + H_2O_2$
Catalase
$H_2O_2$
Superoxide
$O_2^{\cdot -}$
$OH^-$ and $^{\cdot}OH$
$Fe^{2+}$
$^{\cdot}O_2$
Singlet Oxygen
$Fe^{3+} + {}^{\cdot}OH + OH^-$
Hydroxy Radical
R – C = C – R`
Unsaturated lipids in cell membranes
RCOOH +R`COOH
Lipid Peroxides
Vitamine E
Cell Membrane Damage
Chain reactions of Lipid free radicals
$RCOO^- + R`COO^-$
Lipid Free Radical


Brain Antioxidant Defenses
1. Superoxide dismutase: (SOD1 represents as much as 1% SOD of total protein in the brain)
$O_2^{\cdot -}$
$H_2O_2$
Superoxide
Hydrogen Peroxide
2. Catalase: More abundant in astrocytes and white matter than neurons and gray matter.
$H_2O_2$
Catalase
$H_2O$
3. Glutathione (GSH) Peroxidase: GSH peroxidase uses glutathione as a co-factor to convert $H_2O_2$ to water.

## Consequences of Oxidative Stress

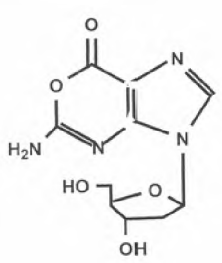
1. Damage to cellular macromolecules
2. Fragmentation of lipids
3. Addition of peroxyl or hydroxyl groups to unsaturated fatty acids
4. Cleavage of fatty acid carbon chain to fatty aldehyde
5. Reaction of fatty aldehydes with free thiol groups to produce thioesters , affecting protein structures and stability
6. Free radicals may cause cross-linking, carbonyl formation, and protein denaturation
7. DNA may be modified, resulting in single-and double strand breaks or miss-parings of purine and pyrimidine during DNA replication.

## Mechanisms of Neuronal Death

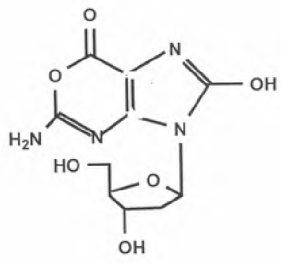
Combined exposure to PB, DEET, and permethrin caused brain cell death via:

1. Impeding the body's ability to metabolize and eliminate test compounds.
2. Increasing the level of reactive oxygen species (ROS), by enhancing their production or reducing the brain's defenses against them.

## A BIOMARKER FOR OXIDATIVE DNA DAMAGE




2'-deoxyguanosine

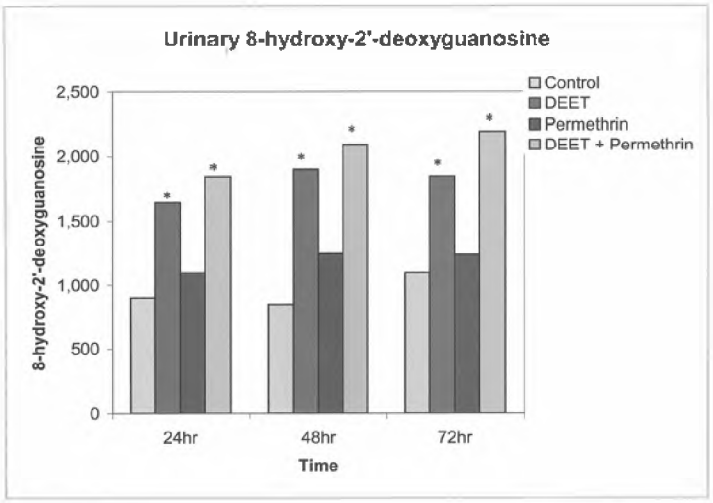



8-Hydroxy-2'-deoxyguanosine

## 8-hydroxy-2'-deoxyguanosine, a Biomaker for Oxidative DNA Damage

1. DEET alone or in combination with permethrin induced excretion of 8-OHdG in rat urine.

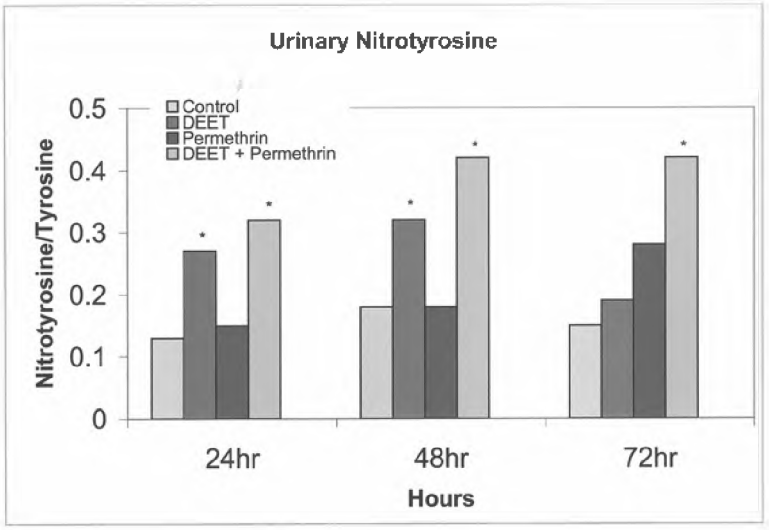
2. Dermal applications of DEET could generate free radical species, causing DNA damage in treated rats.




## 3-nitrotyrosine, a Biomaker for Oxidative Stress

1. A single oral dose of PB or a dermal dose of DEET, alone or in combination significantly increased levels of 3-nitrotyrosine in rat urine.

2. These treatments could generate free radical species, increasing levels of excreted 3-nitrotyrosine.

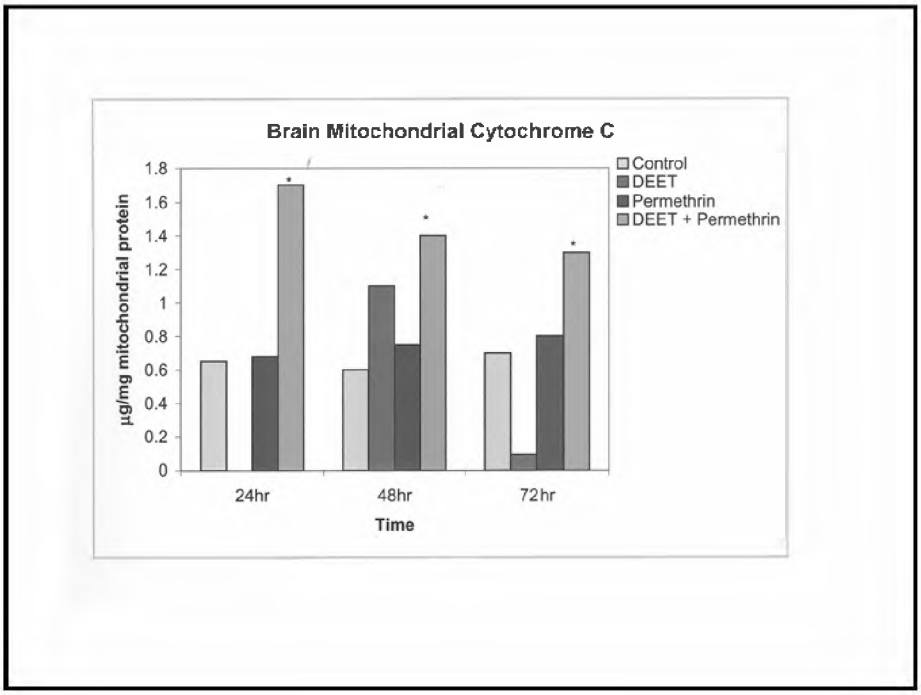



# Brain Mitochondrial Cytochrome c, a Biomarker for Oxidative Stress and Apoptosis

1. A single dermal dose of a combination of DEET and permethrin, significantly increased the release of brain mitochondrial cytochrome c.

2. Neither DEET nor permethrin alone had any effect on mitochondrial cytochrome c of rat brains.

3. Combined exposure to DEET and permethrin could generate reactive oxygen species, leading to an early release of mitochondrial cytochrome c that is involved in apoptotic processes by activating caspases 9 and 3.




# Mechanisms of Neuronal Cell Death following stress chemicals

Chemicals (PB, DEET, Permethrin)

Oxidative Stress

Reactive Oxygen Species (ROS)

+ NO

Peroxynitrile (ONOO·)

DNA

Protein

8-Hydroxy-2'-deoxy-guanosine

3-Nitrotyrosine

Mitochondrial Damage (cytochrome c release; activation of caspases 9,3)

Apoptotic Cell Death

## Neuronal cell Death Consequences

1. Significant death of cerebral cortex neurons results in muscular weakness and loss of strength.

2. A loss of significant amount of hippocampal neurons leads to progressive loss of memory and results in learning disabilities.

3. Loss of Purkinje cells in the cerebellum may cause:
   a. Delays in initiating and terminating movements.
   b. Terminal tremor at the end of the movement.
   c. Disorders in the spatial coordination of hand and finger muscle.

"THESE SYMPTOMS ARE SOME OF THE GULF WAR VETERANS' COMPLAINTS"

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