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Role Played by Mitochondria in Neurotoxic Effects Induced by Polychlorinated Biphenyls

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1. INTRODUCTION

1.1 POLYCLOROBIPHENYLS (PCBs)

Polychlorinated biphenyls (PCBs) are mixtures of up to 209 individual chlorinated compounds (known as congeners) formed by aromatic hydrocarbons and different number of chlorine atoms (Fig.1).

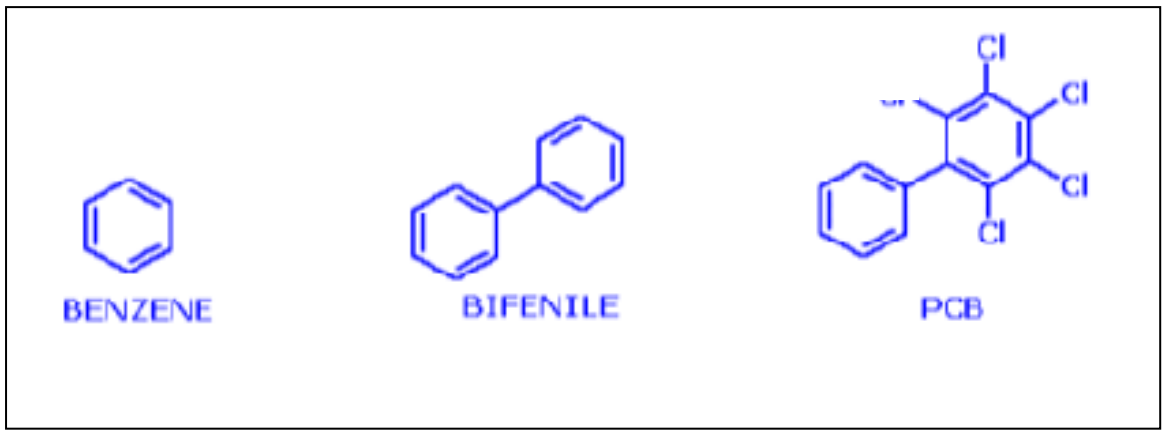


Fig. 1

In the commercial mixtures there are almost 100 of these congeners that are different for the number and for the position of chlorine atoms on the molecule. These differences of composition and structures reflect a different chemical, physics and biological behaviour. PCBs were synthesized, for the first time, by Griefn in 1867 and then were

produced and patented by the USA's chemical company Monsanto in 1929 with the name of Aroclor-derivates. Then, the production was extended in the world, and PCBs were produced in Japan (Kanechlor), Germany (Clophen), Italy (Fenclor e Apirolio) and France (Phenochlor). The company Monsanto commercialized Aroclor 1221,-32,-42,-48,-54,-60,-68.

In 1971, after contamination in the general population, the sale of PCBs in the USA was limited to condensers and transformers. In 1974, there was another further restriction of use of PCBs for domestic applications. Finally in 1976, the use of PCBs was prohibited and the first works for the selling off of PCBs were published by the U.S.A Environmental Protection Agency (EPA) in the 1977.

Between 1930 and 1983 the production of PCBs by the western industry was about 1.5 millions of tons. Presently, 370 tons persist in the environment while the rest is still present, mostly in the electric equipments (*Kannan, 2000*).

PCBs are classified on the basis of the percentage of chlorine in the molecule, since the level of chlorination is important for the property. For this reason the commercial denomination refers to this parameter (Aroclor-1254, for example, refers to a polychlorinated mixtures

containing 54% of weight in chlorine) (*Ballschmiter e Zell, 1980*) (Table 1).

TABLE 1: Characteristics of different mixture of Aroclor

Approximate percentage (w/w) of Aroclors with different degrees of chlorination /WHO,1993)								
Compound	**Chlorine weight(%)**	**Aroclor**						
		1221	**1232**	**1016**	**1242**	**1248**	**1254**	**1260**
	0	**10**						
Monochlorobiphenyl	**18,8**	**50**	**26**	**2**	**3**			
Dichlorobiphenyl	**31,8**	**35**	**29**	**19**	**13**	**2**		
Trichlorobiphenyl	**41,3**	**4**	**24**	**57**	**28**	**18**		
Tetrachlorobiphenyl	**48,6**	**1**	**15**	**22**	**30**	**40**	**11**	
Pentachlorobiphenyl	**54,4**				**22**	**36**	**49**	**12**
Hexachlorobiphenyl	**59,0**				**4**	**4**	**34**	**38**
Heptachlorobiphenyl	**62,8**						**6**	**41**
Octachlorobiphenyl	**66,0**							**8**
Nonachlorobiphenyl	**68,8**							**1**

For the IUPAC nomenclature, the 209 congeners are classified from the n° 1 to n° 209 with increasing order of chlorination. In the commercial mixtures only 130 compouds are present and the majority of these mixtures contains only between 50 and 90 different congeners (*Nicholson e Landrigan, 1994).*

PCBs are stable at different temperatures, resistant to change of pH and to oxidation, provided with a low degree of flammability and are electrically nonconductive, furthermore they possess a Good Heat Exchange.

PCBs are either oily liquids or colorless solids. Some PCBs can exist as a vapor in air and they have no known smell or taste. They have a density greater than water that increase with the chlorination degrade. The point of boiling is approximatly 300 °C at room-pressure, they are highly soluble in organic solvents and in the oils but are less soluble in water, because they have a high relative partition octanel/water coefficient. The important phisical-chemical properties are showed in Table 2.

Table 2: IMPORTANT PHYSICAL CHEMICAL TECHNICALLY PROPERTIES

- easy synthesis
- non flammable
–economical production
- Heat resistant
- chemically stable
- non corrosive
- not crystallize
- continuing high isoelectric
- low vapor pressure
- high boiling
- low solubility in water

1.2 ENVIRONMENTAL PERSISTENCE

Since PCBs are little inflammable and good insulating, they have been used for a long time in the electrical engineering like dielectric liquids or have been used like addictives in transformers, condensers, plasticizers and lubricants.

PCBs have been also largely used for the production of Carbonless Carbon Paper, or like fluids with high heat and pressure resistance.

Actually, the biggest source of contamination are the paints, the condensers and the old piling building (table 3).

In 1986 the prohibition of PCBs use became effective. Despite of this limitation, PCBs are still present in old building and can be released in the environment and inhaled. Moreover, because of the previous contamination and their persistence, now, PCBs are almost omnipresent in the environment.

Table 3: Fields of employment of PCBs

Closed System -transformer -dielectric oil -capacitors -sealant heat -exchanger
Open systems: -cutting oils -greasing -impregnate the fire -softener for plastics
Additive: -toner for photocopiers -glaze -paint

PCBs are an important class of ubiquitous environmental pollutants. Common feature of these mixtures is the persistence in the environment. They completely contaminate the eco-system, indeed,

they are present in the air, in the field, in the water and in the biosphere. They are carried also for long distances or in places where there weren't a real use of these compounds, by the flow waters, by evaporation, by aerosol transport or by the powders. (*Macdonald et al.,2000*) (Table 4).

Table 4: PCB LEVELS IN VARIOUS REGIONS OF THE WORLD (ng/m^3)

Location	Concentration (ng/m^3)
Antarctic coast	0.05-0.2
Canadian Arctic	0.1-0.3
Remote	0.02-0.5
Great Lakes	0.1-5
Rural	0.1-2
Urban	0.5-30
Lake Superior U.S.(peak in spring)	0.2
Lake Superior (low point in fall)	0.065
Various U.S. locations	0.02-36
Marine air	0.05-2
Atlantic Ocean	0.05
Gulf of Mexico	0.2-0.9
North Atlantic Ocean	0.54
West Pacific Ocean	0.84
Bermuda	0.06-1.2
Bloomington, IN	0.7-2.5
North Atlantic Ocean	0.05-1.6
Lake Baikal, Siberia	0.009-0.023
Several oceans and seas	0.004-0.6
Arctic	0.002-0.013
Tokyo, Japan	2
Malsuyama, Japan	2-5
Sweden	<0.8-3.9
Germany	5-10
United States	5
Landfills, U.S	2-18
Electrical substations U.S	1-47
Transformer manufacturer, U.S.	17
Spill site, U.S.	10-10.8

In many uncontaminated places, the quantity of PCBs is about 0,003ng/m^3, in contaminated places is up to 3 ng/m^3.

The concentrations of PCBs are particularly high in the soil where continuously they redistribute. PCBs reach the soil from the atmosphere, for deposition of the dust or through precipitations, the superficial water and the plants (Table 5).

Table 5: PERSISTENCE OF PCBS IN AIR, WATER AND SOIL

Compounds	**Air**	**Water**	**Soil**	**Sediment**
Monochlorobiphenyl	~ 1week	~ 8 months	~ 2 years	~ 2 years
Dichlorobiphenyl	~ 2 week	~ 8 months	~ 2 years	~ 2 years
Trichlorobiphenyl	~ 3 week	~ 2 years	~ 6 years	~ 6 years
Tetrachlorobiphenyl	~ 2 months	~ 6 years	~ 6 years	~ 6 years
Pentachlorobiphenyl	~ 2 months	~ 6 years	~ 6 years	~ 6 years
Hexachlorobiphenyl	~ 8 months	~ 6 years	~ 6 years	~ 6 years
Heptachlorobiphenyl	~ 8 months	~ 6 years	~ 6 years	~ 6 years
Octachlorobiphenyl	~ 2 years	~ 6 years	~ 6 years	~ 6 years
Nonachlorobiphenyl	~ 2 years	~ 6 years	~ 6 years	~ 6 years
Decachlorobiphenyl	~ 6 years	~ 6 years	~ 6 years	~ 6 years

Like reported in table 5, a different PCBs distribution is in the environment, according to the chlorination degrade. Indeed, the biphenyls with one o less chlorine atoms are in the atmosphere; the biphenyls with 1-4 chlorine atoms migrate towards the polar latitude; the biphenyls with a number of chlorine atoms between four and eight are in the middle latitude, and the biphenyls with more than eight chlorine atoms remain near the ‘’sources’’ of contaminants (*Wania e Mackay, 1996*). The number of chlorine atoms is associated with a bigger stability and a smaller diffusion. **Biodegradation:** the aerobic and anaerobic degradation of PCBs is a very important process but it happens very slowly. The degradation of PCBs congeners depends on many factors like: the initial concentration, the humidity, and temperature (high temperatures increase the degradation), or on the presence of suppressant compounds (chlorine-benzoates) and on the availability of bacterial nutrients like carbon source.

The anaerobic way is involved in the degradation of highly chlorinated PCBs, the aerobic way is effective with congeners with low chlorination. In the air the PCBs are above all like steam. They are modified by UV photolysis and can be transformed in others toxic compounds. In the water the degradation is anaerobic and happens by photolysis. In the soil some of PCBs can be partially degraded by bacteria organisms.

The biodegradability decreases with the chlorination, and the congeners with more than five chlorine atoms are resistant to degradation, by contrast the congeners with more than three chlorine atoms persist in the environment for long time.

All the commercial mixtures contain many congeners with different chlorination, so we can affirm that all the PCB mixtures are highly persistent in the environment. Although the PCBs use in new products have been reduced to the minimum in all the country, the PCBs produced until 1980 continue to redistribute into environmental compartments. They are still released by the not correctly discarded scraps, and then found in the soil. (*ATSDR. 2000*).

1.3 UPTAKE, METABOLISM AND ELIMINATION

Many studies showed that the PCB congeners are adsorbed mostly in the gastrointestinal tract by passive diffusion *(Schecter A. et al.,1993; ATSDR. 2000*). This absorption increases with the increase of chlorination. In the blood, the PCBs bind to the lipoprotein fraction, and accumulate in this tissue and persist for years (*ATSDR 2000*).

The PCBs can be metabolized, and the products of metabolism can touch off biochemical and toxicological effects (*Safe, 1994*). The

different congeners, with different steric configuration are metabolized with different enzymatic pathways (*ATSDR 2000*).

They are metabolized mostly in the liver by the cytochrome P-450 monooxygenase, with a different velocity depending on their chlorination and on the position of chlorine atoms. The PCB's metabolism is isozyme-specific. Both, the PCBs mixtures and the single PCBs congeners are able to activate the enzyme with catalytic activity, in the same manner of phenobarbital and metylcolantrene.

The PCBs are also able to activate the phase II-enzyme, like the glutathione-S-transferasy (GST) and the glucuronyl-transferasy (UGT). These metabolic reaction improve the polarity of PCBs and also their elimination (*Safe,1994*).

The congeners with a lower chlorine degree are eliminated by the urine, while the highly chlorinated congeners are biologically persistent (*ATSDR. 2000*). Also the feeding represents an important elimination way.

The mechanism throught the coplanar PCB's cause toxicological effecte is bound to their affinity with the cytosolic Ah receptor. This receptor regulates the activity of aryl-hydrocarbon hydroxylase enzyme (AhH) that induces the genomic transcription of phase 1-enzyme CIP 1A1 and phase 2- enzyme GST and UGT (fig. 2).

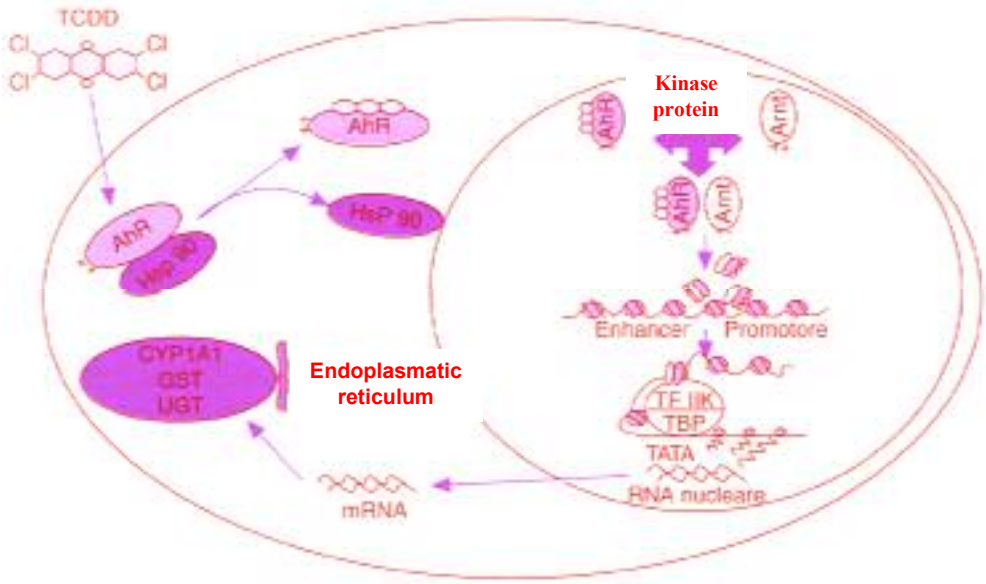


Fig.2

The PCBs are slowly metabolized in the mammal organism. Because of their high solubility in the lipids and of their slow elimination, they accumulate in the adipose tissue of animals and of humans. The population is exposed to PCBs primarily by the use of fish, meal and chicken foods (*ATSDR. 2000*). The scientist tried to quantify the PCBs contamination by food, but it is quite complicated (*Danse et al. 1997*). *Duarte-Davidson* e *Jones* study (1984) estimated that in Britain the 97% of exposition is by food, the 3,4% is by the air and 0,04% by the water. Moreover, in USA, the intake of fish is one of most source of contamination, mostly in the children between 1 and 5

years old. Also the shellfishes contribute to the food exposition to PCBs, while the milk and the meal are less important (less than 5% of the total average exposition) (Table 6).

Table 6: AMOUNT OF PCB IN FOODS

Test material	PCB mg /kg	Sample
Arable land	0,03	Dry substance
Deposit filtration	0,75	Dry substance
Plant food	0,009	Fresh substance
Cereals	0,005+0,12	Fresh substance
Vegetables	0,005	Fresh substance
Margarine	0,013	Fresh substance
Sea fish	0,12+0,33	Fillet weight
Fathead minnow	0,74+1,01	Fillet weight
Eel	1,43+6,51	Alive weight
Beef	0,067	Fat fraction
Pork	0,069	Fat fraction
Cold cuts	0,62	Fat fraction
Animal fat	0,23	Fat fraction
Milk	0,09+0,14	Fat fraction
Butter	0,11	Fat fraction
Cheese	0,07	Fat fraction

1.4 HEALTH EFFECTS

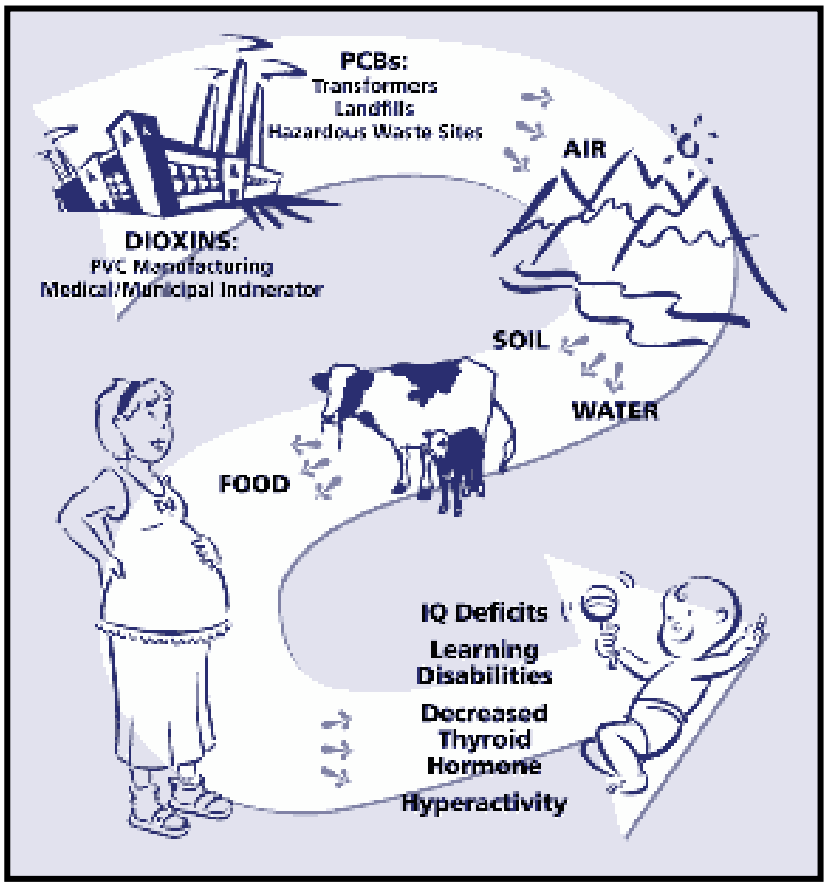


Fig.3

It is known that PCBs accumulate in the foetal tissue. Indeed, they of PCBs are able to pass the placenta barrier because of their high solubility in lipids.

Also the feeding represents one of the most PCBs congeners elimination way.

To understand the level of PCBs contamination and their toxicological effects is almost complicated now, mostly because it is difficult to discriminate between the PCBs effects and the toxicological effects of

others chemical products present in the environment (*Danse et al., 1997; Connon et al., 1998*).

The most part of toxicological study refers to data obtained from samples collected from workers exposed to high quantity for a long time, or to events of common poisoning.

It has been possible to distinguish three groups of people differently exposed to PCBs contamination. The first group includes people exposed to industrial contamination, where the exposition happens mostly by inhalation and skin uptake. The second group includes the Japanese and Taiwan people who had eaten contaminated rice oil.

In Japan, in 1984 there was a very important PCBs contamination, called ‘’Yuosho’’, oil-illness in Japanese, that damaged about 1800 persons (*Kuratsune and Shapiro,1984*).

In 1979, in Taiwan there was a similar event of contamination, and about 2000 persons were affected by ”Yu Cheng”, Chinese oil-illness (*Kuratsune and Shapiro, 1984*).

Finally, the third group is composed by the rest of worldwide population continuously exposed to the PCBs by food.

Mostly the newborns are at the risk of contamination drinking breast milk of mothers which have taken big quantity of contaminated fish and meal.

The studies of toxicological and clinical effects of PCBs are particularly difficult because the PCBs are used mostly like more congener mixtures and not like single substances. Moreover, the toxicological studies have to take into account many factors like the influence of others pollutants, or the people samples life style, the environmental factors, the socio-economic factors, the genetic factors and the mother's age during pregnancy.

Many studies showed that PCBs cause endocrinological and reproduction's damages (*Shantz et al 1996*). Children born by women exposed to PCBs, frequently are affected by numerous pathologies like skin and oral cavity hyperpigmentation, gingival hyperplasia, bones and dental alterations.

Repeated PCBs administrations can cause liver cancer in rats, and also in this case, the gravity of pathology is tightly correlated to the composition of PCBs mixtures (*Ahlborg et al., 1991*).

The PCBs mixtures are not complete carcinogens but they can be cancer promoters (*Ahlborg et al.,1991; Safe,1994*).

In Yusho and Yu-Cheng patients many deaths cases due to cancers have been found.

Many epidemiological studies made on workers exposed to high PCBs concentrations presupposed a possible correlation between the lymphatic, hematopoietic and gastroenteric system damages and the

PCBs exposition, but at the moment there aren't clear data about this issue.

In Yusho and Yu-Cheng patients have been also found serious dermatological alterations like chloracne and skin, gingival and nails hyperpigmentation, lasted for a long time, approximately 10-14 years after the contamination (*Kimbrough, 1995; Longnecker et al., 1997*).

1.5 EFFECTS ON CENTRAL NERVOUS SYSTEM

The increase of neuronal damages by PCB's exposition drove the scientists to make epidemiological studies and on animal models to evaluate the neurotoxic effects of PCBs. In particular, have been carried out studies on children accidentally exposed to PCBs. Interesting data on neurotoxicological effects of PCBs have been leaded by epidemiological studies carried out in North Caroline, in women who had consumed fish from Michigan lake, and in Japan and in Taiwan in women who ingested contaminated rice oil. These studies suggested that PCBs prenatal exposition associated to others toxics, affects negatively the offspring's neurological development, causing motor and learning diseases (*ATSDR. 2000*). In some cases, data were compromised by the difficulty to dissociate between the PCBs effects and those of dioxin .

The most important incidents with a PCBs contamination occurred in Japan and Taiwan in 1968 and 1979, wherein 4000 peoples ingested contaminated oil (*kuratsune et al., 1971; Hsu et al., 1985*).

In these two cases, the children exposed in prenatal period or during the feeding showed motor deficits and cognitive impairments (*Seegal, 1996*). Others studies show learning changing in children exposed to low doses of PCBs during the embryogenesis, demonstrating that the embryonic exposure is more toxic than the simple feeding (*Rogan & Gladen, 1992; Jacobson & Jacobson, 1996*).

The most common effects of PCBs exposition, during the development, are hyperactivity, learning and memory deficits while after adult exposition motor activity reduction is observed (*Tilson et al., 1990; Seegal, 1996; Giesy & Kannan, 1998; Widholm et al., 2001*). Recent studies suggested that the PCBs exposition is a cause of alterations in the release of neurotransmitters such as catecholamines (*Shain et al, 1991*) and it can alter the long-term potentiation phenomenon (*Altmann et al., 1995; Gilbert & Crofton, 1999*).

In animal models the PCB mixtures caused learning impairments in rats exposed in the prenatal period, but there aren't learning changes in rats exposed during the postnatal period (*Ahlborg et al.,1992; Safe,1994*). Behavioural studies performed on Rhesus monkeys,

proved that the offspring come from monkeys exposed to PCBs, developed hyperactivity, learning retardation, and cognitive impairment (*Ahlborg et al.,1992; Seegal,1996*).

1.6. CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN THE PCBs NEURO-TOXICOLOGICAL DAMAGE

Even if there is a clear evidence of a correlation between the neurological damage and the PCBs exposition, the molecular and the cellular mechanisms implicated in these alterations are not completely clear. The neuro-toxicological effect has been correlated with the alteration of the neurotransmitter release, with the increase of the production of ROS and with the increase of cytosolic calcium. It has also been demonstrated that the exposition to PCBs, in particular to the ortho-substitutes, could cause the decrease of dopamine levels in different brain's areas in rats and primates (*Seegal, 1996*). This effect can be caused by the inhibition of the dopamine uptake at vesicular level by PCBs (*Mariussen et al., 1999*).

In prepared synaptosomes, low concentrations of PCBs, inhibited reuptake of neurotransmitters like GABA and glutamate *(Mariussen & Fonnum, 2001*). Therefore, the increase of extracellular glutamate could have a major role in the excitotoxicity by PCBs.

During cell death process, calcium ions play a very important role. In this regard It has been reported that the Polychlorinated biphenyls are able to modify the calcium homeostasis in different subcellular compartment like synaptosomes, mitochondria and microsomes, and cerebellum granules (*Tilson & Kodavanti, 1997; Wong et al., 1997; Mundy et al., 1999; Inglefield & Shaker, 2000*).

PCBs can increase the $[Ca^2]i$ in many ways. Very important for this increase is the Ca^2 influx through the L- Ca^{2+} voltage channels (VSCCs) (*Inglefield & Shafer, 2000*). Others studies showed that the PCBs exposition can increase the Ca^{2+} release from the intracellular stores (*Inglefield at al 2001*). Recently it has been also demonstrated that the PCBs can activate the sodium-calcium exchanger in its reverse-mode causing the increase of entrance of calcium through this membrane pump (*Magi et al., 2005*).

Previous studies performed in our laboratory demonstrated that the PCBs cellular damage in neuroblastoma cells was linked to the increase of cytosolic calcium. This increase caused the activation of a chain of transductional events stimulating the increase of expression of the beta isoform of nNOS protein. From other studies, it seems that this up-regulation occurs in response to the neuronal oxidative damage (*Catania et al, 2001*).

The nNOS up-regulation, in SH-SY5Y neuroblastoma cells exposed to the PCBs mixture Aroclor-1254, was accompanied by the NO production increase, and by the consequent activation of GC/cGMP/PKG pathway (*Canzoniero LM et al., 2006)*.

Hence, these studies suggest that the Ca^{2+}-dependent nNOS activation causes the increase of NO release which, by the GC/cGMP/PKG pathway activation, could be involved in the cellular events that lead to PCB induced cell death. (fig.4)

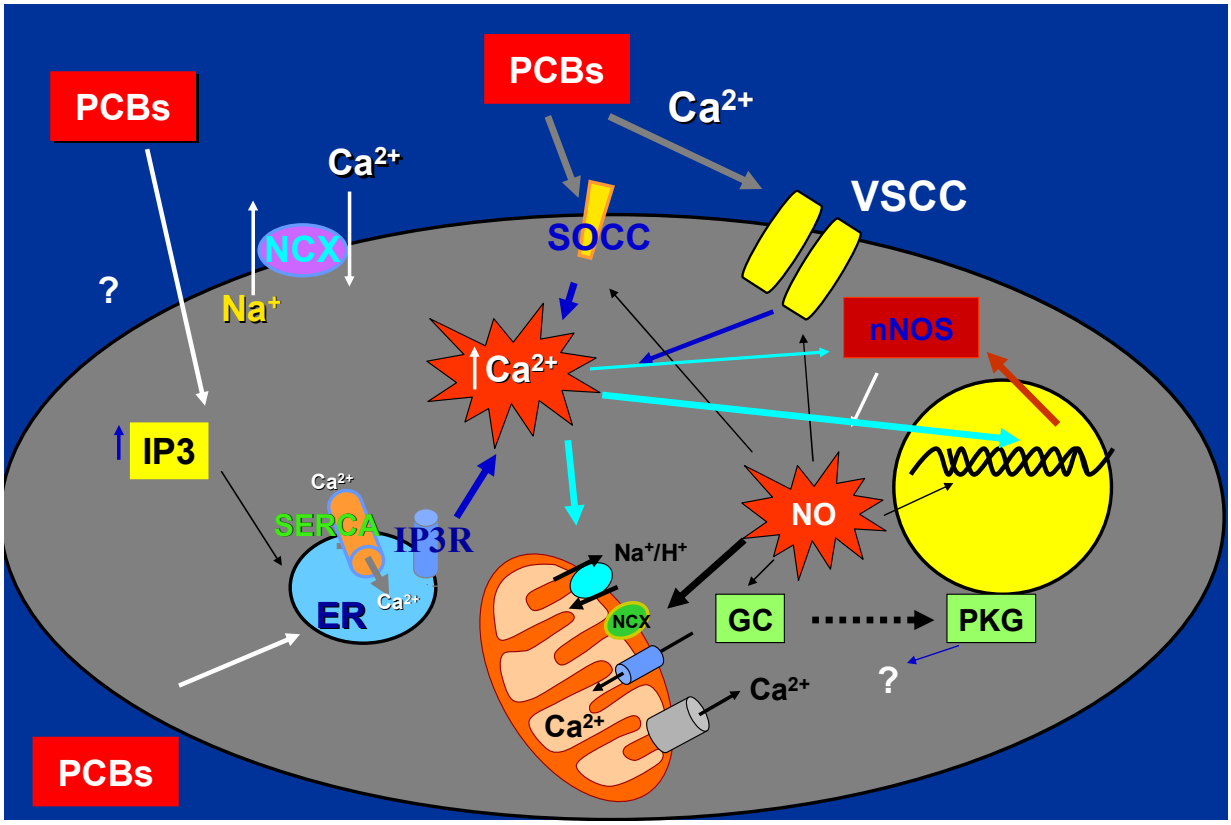


Fig.4: TRANSDUCTIONAL PATHWAYS INVOLVED IN PCB ACTION

Furthermore since it has been suggested that NO could exert toxic effects on mitochondria the increase induced by PCBs of NO production suggests the possibility that PCB-induced injury might involve mitochondria . Indeed, the increase of NO into the mitochondria and the consequent peroxynitrite production could impair the mitochondrial respiratory chain *(Christoph Richter, 1997).* Nitric oxide (NO) and peroxynitrite (ONOO) avidly interact with mitochondrial components, leading to a range of biological responses spanning from the modulation of mitochondrial respiration, mitochondrial dysfunction to the signaling of apoptotic cell death. Physiological levels of NO primarily interact with cytochrome c oxidase, leading to a competitive and reversible inhibition of mitochondrial oxygen uptake. In turn, this leads to alterations in electrochemical gradients, which affect calcium uptake and may regulate processes such as mitochondrial transition pore (MTP) opening and the release of proapoptotic proteins. Large or persistent levels of NO in mitochondria promote mitochondrial oxidant formation. Peroxynitrite formed either extra or intramitochondrially leads to oxidative damage, most notably at complexes I and II of the electron transport chain, ATPase, aconitase and Mn-superoxide dismutase. Mitochondrial scavenging systems for peroxynitrite and peroxynitrite derived radicals such as carbonate ($CO_3^{\cdot}$) and nitrogen

dioxide radicals (NO2) include cytochrome c-oxidase, glutathione and ubiquinol serve to partially attenuate the reactions of these oxidants with critical mitochondrial targets. Detection of nitrated mitochondrial proteins in vivo supports the concept that mitochondria constitute central loci of the toxic effects of excess reactive nitrogen species *(Radi R, et al, 2002).* Recent studies showed that the increase of $[Ca^{2+}]_i$ and the reduction of mitochondrial membrane potential followed by the increase of ROS production, are involved in the activation of transductional mechanisms leading to the cell death (*Ham YM et al,. 2006*)

2. AIM OF THE STUDY

The general aim of this research has been to evaluate the role of mitochondria in the neurotoxic effects played by PCBs. Mitochondria are the powerhouse of the cell and their primary physiological function is to generate adenosine triphosphate (ATP) through oxidative phosphorylation via the electron transport chain. These

organelles generate energy primarily in the form of the electrochemical proton gradient (ΔμH+), which fuels ATP production, ion transport, and metabolism. Generation of this universal energy currency, ΔμH+, occurs through the series of oxidative reactions conducted by the respiratory chain complexes at the level of ion-impermeable, almost cholesterol-free inner membrane. Reduced nicotinamide adenine dinucleotide represents the entry point to the complex I (reduced nicotinamide adenine dinucleotide: ubiquinone reductase), whereas the reduced ubiquinol enters the respiratory chain in the complex III (ubiquinol: cytochrome *c* (cytc) reductase) to reduce cyt-c, the electron carrier to the complex IV, cyt-c oxidase. Each of these steps generates ΔμH+ by pumping protons from the mitochondrial matrix to the intermembrane space and is coupled to electron flow, thus generating the electric membrane potential of −180 to −220 mV and a pH gradient of 0.4 0.6 U across the inner mitochondrial membrane resulting in the negatively charged matrix side of the membrane and alkaline matrix. Ultimately, accumulated ΔμH+ is converted into the influx of protons into the matrix driving ATP synthesis or protein transport. In addition, these end points are necessary for the execution of 2 major enzymatic metabolic pathways within the mitochondrial matrix: the tricarboxylic acid (TCA) oxidation cycle and the fatty acid β-oxidation pathway.

This intricate system fueling cellular functions is as elegant as it is vulnerable: practically every component of the system, from the electron transport chain complexes to the permeability properties of the membranes, can be a target for various noxious stimuli, some of which can be generated within mitochondria themselves *(Addabbo F et al., 2009)*. The central nervous system is particularly vulnerable to oxidative damage due to its high energy expenditure and oxygen demand. Elevated concentrations of free radicals and resultant oxidative damage, such as lipid peroxidation and protein carbonylation, have been repeatedly demonstrated in neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and ischemic stroke *(Andersen, 2004*). Recent studies have shown that mitochondrially-formed oxidants are mediators of molecular signaling, which is implicated in the mitochondria-dependent apoptotic pathway that involves pro- and antiapoptotic protein binding, the release of cytochrome c, and transcription-independent p53 signaling, leading to neuronal death. Oxidative stress and the redox state of ischemic neurons are also implicated in the signaling pathway that involves phosphatidylinositol3-kinase/Akt and downstream signaling, which leads to neuronal survival.

PCBs, increasing the nNOS expression and its function cause the increase of NO production which can generate other reactive nitrogen species (RNS). Although excess ROS and RNS can lead to oxidative and nitrosative stress, moderate to low levels of both can influence cellular signaling pathways. Thus particularly important are the roles of these mitochondrially generated free radicals in hypoxic signaling pathways, which have important implications for cancer, inflammation and a variety of other diseases.

On the other hand mitochondria, which generate ATP through oxidative phosphorylation to meet cellular energy demand are among the main targets of ROS (reactive oxygen species) and NOS (nitrogen species). These compounds are highly unstable and cause oxidative damage to cellular proteins, lipids and nucleic acids, and especially of the mitochondria where they cause membrane damage of mitochondrial respiratory chain enzymes determines changes in ATP production.

Several antioxidants have been used to prevent cellular damage induced by ROS and RNS. Among antioxidant compounds, particolarly interesting is the effect exerted by pyruvate. Inside the cell, the pyruvate is the end product of glycolysis, obtained by dephosphorylation of phosphoenolpyruvate, and is produced in the rate of two molecules for each glucose molecule introduced into the

process. Pyruvate is also a possible starting compounds for gluconeogenesis, the process leading to the formation of molecules of glucose when needed by the cell. Overall, then it is an intermediate element in the anabolic process and into the catabolism of carbohydrates. As a product of glycolysis, pyruvate is transported into the mitochondria where it undergoes a series of reactions catalyzed by pyruvate dehydrogenase, which transforms it in acetyl-CoA (acetyl coenzyme A) and at the same time releasing a molecule of carbon dioxide and reducing one molecule of NAD^+ to NADH. At this point the acetyl-CoA is ready to be used in the Krebs cycle or citric acid cycle. At the end of glycolysis, the pyruvate can also be used to produce the aminoacid alanine in a reaction catalyzed by alanine aminotransferase. In anaerobic conditions the pyruvate can be converted to lactic acid by lactate dehydrogenase (which in biological systems is in the form of lactate anion) in the lactic fermentation, or ethanol in alcoholic fermentation by two enzymes: pyruvate decarboxylase and alcohol dehydrogenase (ADH). When molecules of glucose are necessary, pyruvate is used in the process of gluconeogenesis, generating new glucose. The molecule may enter into this process as it is, thereby representing the first stage of the process itself, but it may also be the second stage, where it is the product of the transformation of alanine or lactate in pyruvate. In both

case is converted to oxalacetate by the enzyme pyruvate carboxylase. (fig.5)

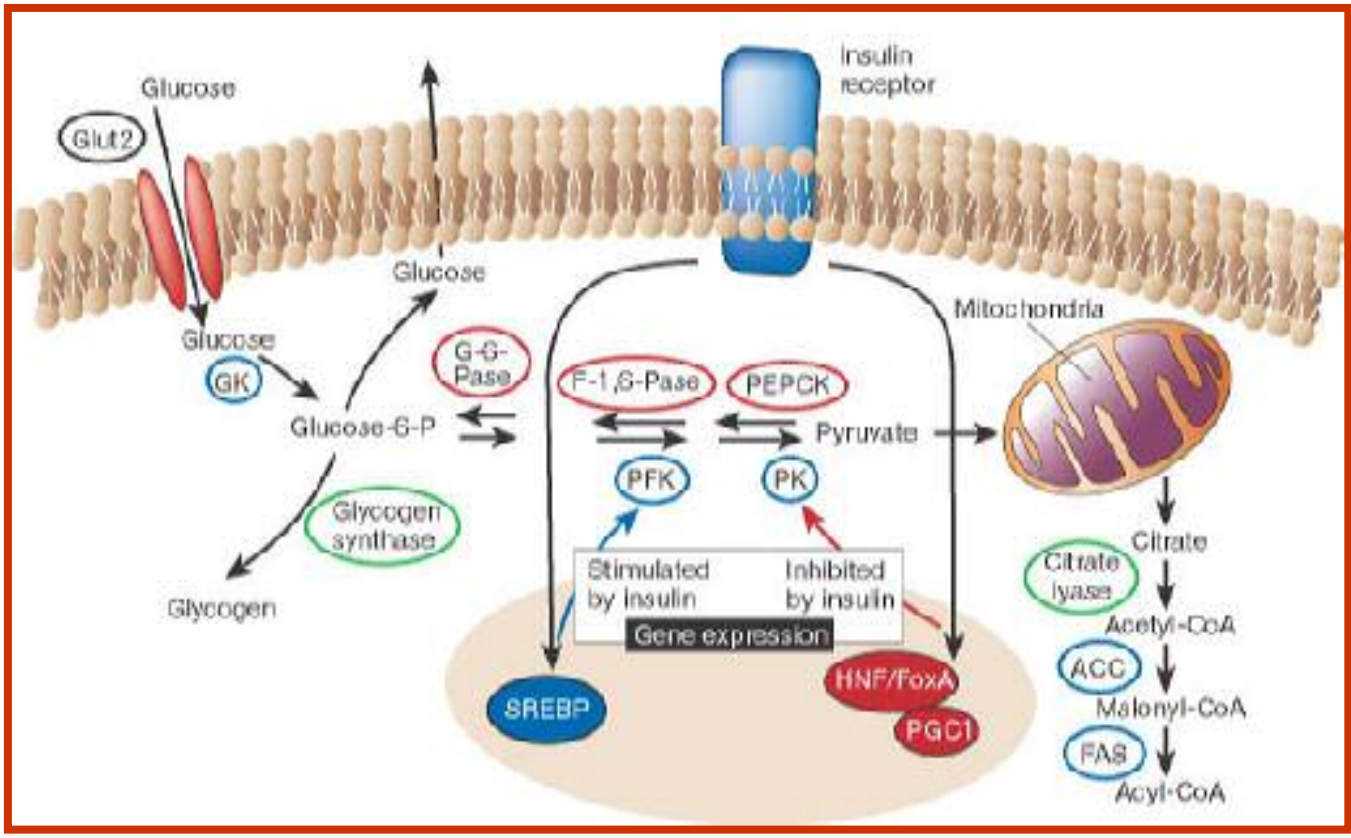


Fig.5. AEROBIC SYNTHESIS OF ATP: PATHWAY DIAGRAM

Its use (as such but the ache as sodium) and usefulness in research labs is due to the large number of biochemical processes in which it is involved both as substrate and as allosteric modulator. Furthermore, pyruvate has antioxidant properties due to its keto-carboxylic acid part of the molecule that directly neutralizes peroxides and peroxynitrite (*Maller et al. , 2005).* In several models

"in vitro" and "in vivo" it has been shown that pyruvate has a neuroprotective role against oxidative stress. Indeed, in neuronal cell cultures, pyruvate protects against various insults *such as* β-*amyloid (Alvarez et al., 2003), H2O2 (Yoo et al., 2004*), mitochondrial toxins (*Mazzio and Solimen, 2003b*) and zinc (*Chen and Liao, 2003*). Furthermore in *In vivo* models pyruvate protects against damage from cerebral ischemia (*Lee et al., Mongan et al., 2001*) and toxicity of zinc (*Lee et al., 2001*).

For these reasons the second aim of this work has been to evaluate the neuroprotective effect of pyruvic acid, and in particular its antioxidant role during PCB exposition. Experiments of the present study have been performed on human neuroblastoma SH-SY5Y cells, which possess characteristics similar to those of dopaminergic, cholinergic, glutamatergic and adenosinergic neurons and therefore are widely used to study cellular and molecular mechanisms of neuronal patthophysiology.

These cells were properly exposed to the polychlorinated biphenyls mixture Aroclor-1254, and the following parameters were evaluated:

1) Cell viability evaluated as mitochondrial activity that was quantified the MTT assay, which measures the activity of mitochondrial

dehydrogenases to convert the tetrazolo salts in insoluble formazano salts.

2) Mitochondrial activity evaluated in terms of changes in mitochondrial membrane potential and ATP production. Furthermore the effect of PCB exposure on the activity of each enzyme complex of the mitochondrial respiratory chain by ‘’Blue Native PAGE’’, a technique that allows to separate the mitochondrial respiratory chain complexes in their active form and to assess their activity in vitro through specific protocols for staining, was also evaluated.

3) The effect of pyruvate, an energy substrate with antioxidant activity on cell and mitochondrial toxicity induced PCB exposure

3. MATERIALS AND METHODS

3.1. CELL CULTURE

The cells are SH-SY5Y human neuroblastoma cells that were obtained from the manufacturer (American Type Culture Collection, VA). The cells awerecultured in RPMI (10% inactivated fetal bovine

serum and penicillin / streptomycin) at 37 ° C, 5% CO2 and 95% air in an incubator for cell culture.

3.2. ENVIRONMENTAL POLLUTANTS STUDIED

Aroclor-1254 (Supelco) mixture containing several PCB congeners, ortho and para-substituted.

3.3 TREATMENTS

The human neuroblastoma cells SH-SY5Y were exposed for 24h to different concentrations (1, 5 and 10 μM) of the mixture Aroclor-1254 l in RPMI culture medium devoid of fetal calf serum in order to establish a dose-related effect on cell viability. In the studies of the effect of PCBs on mitochondrial activity, SH-SY5Y cells were treated with the mixture Aroclor-1254 at a concentration of 5μg/ml, that represents the EC 50 detected in the previous experiments for 6 hours. In both studies, the pyruvate, in the form of sodium salt, was added to the culture medium and used in a concentration range of 1 to 10 mM.

3.4 DETERMINATION OF CELL VIABILITY

Cell death was evaluated by measuring the ratio between dead and living cells. To quantify cell death, after experimental procedures, SH-SY5Y cells were washed with normal Krebs and double stained with 36 μM fluorescein diacetate (FDA) and 7 μM propidium iodide (PI) for 20 min at 37° C in a phosphate buffer solution. Stained cells were examined immediately with a standard inverse fluorescence microscope at 480 and 546 nm *(S. Amoroso et al 1999).* PI positive and FDA positive cells were counted in three representative high power fields of independent cultures and cell death was determined by the ratio of the number of PI positive cells/PI + FDA stained positive cells *(Wei H. et al. 2000).*

3.5 DETERMINATION OF MITOCHONDRIAL ACTIVITY

Mitochondrial dysfunction was evaluated with (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) (MTT) test [1,2], in which the MTT dye is metabolized by viable mitochondria to a colored product and can be detected photometrically. Briefly, after the experimental procedures, SH-SY5Y cells were washed with normal Krebs and incubated with 1 ml of MTT solution (0.5 mg /ml in PBS). This yellow water-soluble tetrazolium salt is converted into a water-insoluble

purple formazan by the succinate dehydrogenase system of the active mitochondria. Therefore, the amount of formazan produced is proportional to the number of cells with mitochondria that are still vital [1,2]. After 1 hr incubation at 37° C, cells were dissolved in 1ml of dimethylsulphoxide (DMSO), in which the rate of MTT reduction was measured using a spectrophotometer at a wavelength of 540 nm. Data are expressed as percentage of mitochondrial dysfunction versus sham-treated cultures.

3.6 DETERMINATION OF MITOCHONDRIAL MEMBRANE POTENTIAL

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed using the fluorescent dye tetramethyl rhodamine ethyl ester (TMRE) in the 'redistribution mode' as described previously (*Livigni et al, 2006*). Confocal images were obtained using a Zeiss inverted 510 confocal laser-scanning microscope and a × 63 oil-immersion objective. The illumination intensity of 543 mm xenon laser, used to excite TMRE fluorescence, was kept to a minimum (0.5%) of laser output in order to avoid phototoxicity.

3.7 QUANTIFICATION OF ATP CONTENT

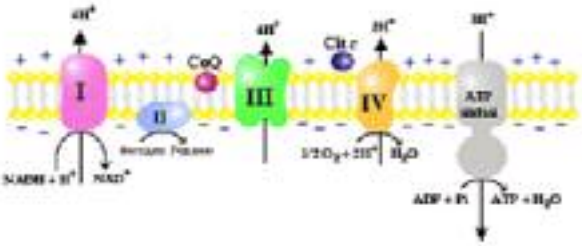
ATP content was measured with a commercial bioluminescent assay (ATP bioluminescent assay kit, Sigma, St Louis, Missouri, USA) according to manufacturer's instruction. Briefly, ATP was extracted by boiling the samples in a solution containing (in mM) 100 TRIS, 4 EDTA, pH 7.75. After centrifugation at 10 000 X g for 60 s, samples were diluted at 1:50 in dilution buffer (Sigma, FL-AA). To obtain bioluminescence measurements with a standard luminometer, 100 µl of supernatant was mixed with 100µl of luciferin-luciferase solution. The standard curve of ATP was obtained by serial dilution of 2 µM ATP solution *(Maeda M. 2003).*

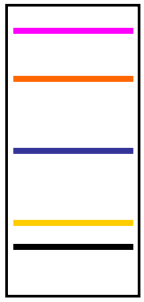
3.8 BLUE NATIVE PAGE

This is a technique for separating the mitochondrial respiratory chain complexes in their active form to assess their activity in vitro through specific protocols for staining. As described by Schägger and von Jagow (1991), the mitochondrial respiratory chain complexes in their native form are extracted from a cell pellet weighing about 10mg. This protocol of extraction is to use the colored compound SERVA Blue G, also known as Coomassie Blue G250. This molecule gives a charge to the protein complexes: thus electrophoresis, performed in native

conditions, which allows proteins to be separated solely based on their net charge (electrophoresis gel under non-denaturing). Generally used gel 6-13% polyacrylamide gradient determining the proper separation of proteins on the molecular weight between 100 and 1000Kda.(fig.6)

Complex





I (Monomeric)……750kDa
V (Monomeric)…..600kDa

III (Dimeric)……500kDa

IV (Monomeric-Dimeric)..200kDa
II (Monomeric)……..130kDa

Fig.6

At the end of the run electrophoresis gels are incubated with specific substrates and reagents solutions glad of their respiratory complexes (table 7).

Complex and Activities	Buffer	pH	Substrates	Reagents
Complex I: NADH dehydrogenase	Tris-HCl	7.4	NADH	NTB
Complex II: Succinic dehydrogenase	KH_2PO_4 ATP PMS	7.4	Succinate	NTB
Complex IV: Cytochrome C oxidase	KH_2PO_4	7.4	Cytochromo C	DAB
Complex V: ATPasi	Glicina MgCl2	8.6	ATP	$Pb(NO_3)_2$

At the end of the incubation period is possible to observe the reactions of the complex bye the colorimetric reaction denoting their activities.

In particular, the dehydrogenase activity on complex I is highlighted by the NTB reduction to formazan which precipitates in the gel in place of the corresponding enzyme, resulting in a purple band. The succinic dehydrogenase, catalyzing the NTB reduction in the presence of a carrier of electrons which the LDCs also results in the precipitation of the compound formazan. The activity of cytochrome c oxidase is evidenced by oxidation of DAB by the cytochrome C. Finally, the ATPasi activity is evident following the precipitation of inorganic phosphate originating from ATP hydrolysis. (fig.7)

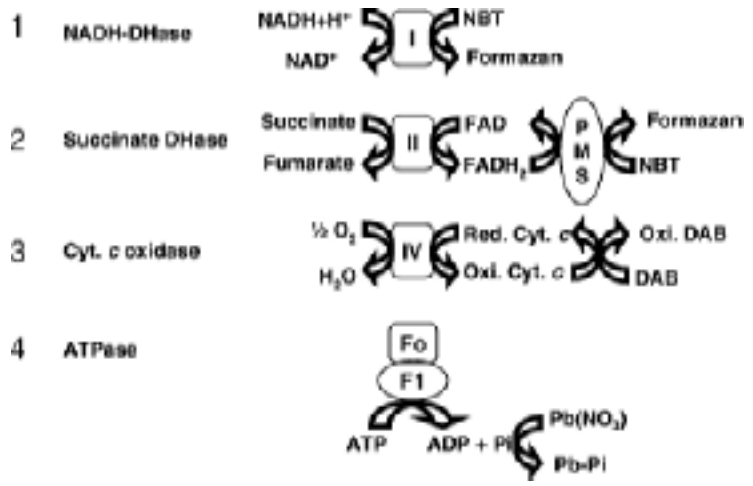


FiG.7 MITOCHONDRIAL COMPLEXE S ACTIVITIES

4. RESULTS

4.1 EFFECT OF DIFFERENT CONCENTRATIONS OF PCB ON CELL VIABILITY

Exposure to the mixture of PCB Aroclor-1254 at different concentrations (1-5-10 µg/ml) for 24 hours caused a dose related cell death (Fig 8).

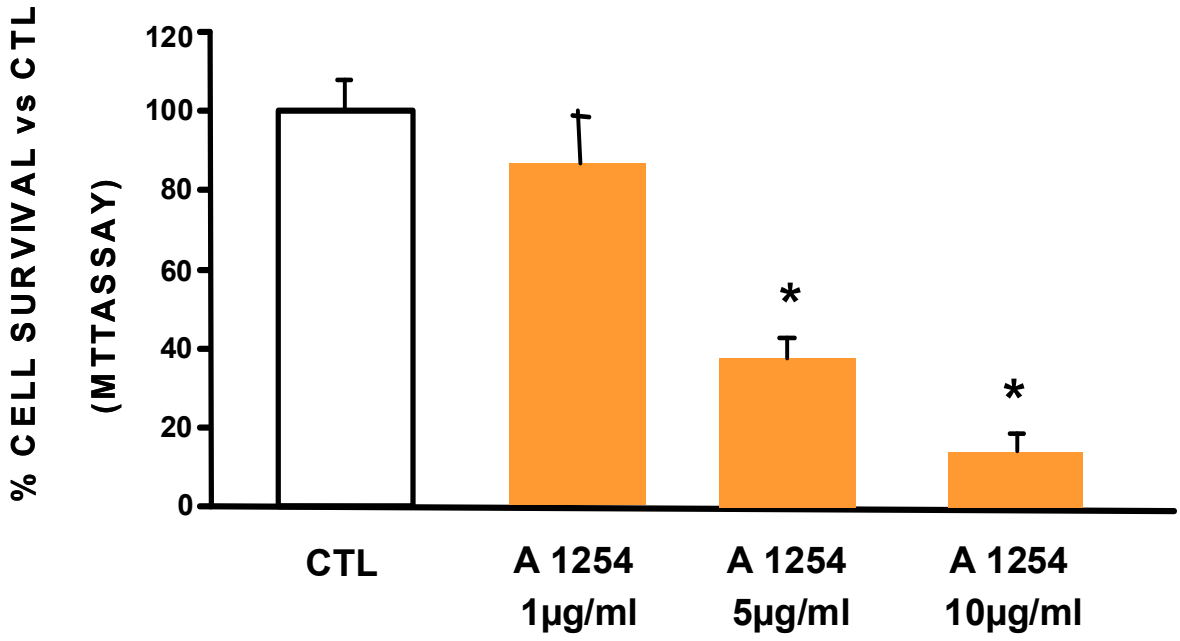


Fig.8: Effect of Aroclor-1254 in mitochondrial oxidative activity after 24hrs exposure SH-SY5Y cells. Each bar represents the mean 6 SEM (10 separate experiments) of cell viability assessed by MTT assay. *P < 0.05 vs control group (CTL)

4.2 EFFECT OF AROCLOR ON MITOCHONDRIAL ACTIVITY

Mitochondrial suffering was evaluated in terms of membrane potential and ATP production. The membrane potential was studied by confocal microscopy techniques by the use of fluorescent probes sensitive to the variation of this potential. At the concentration of 5 µg/ml, that represents the concentration able to induce approximately a 50% cell damage, Aroclor-1254 induced mitochondrial suffering after an incubation period of 6 hours.

(Fig.9-10)

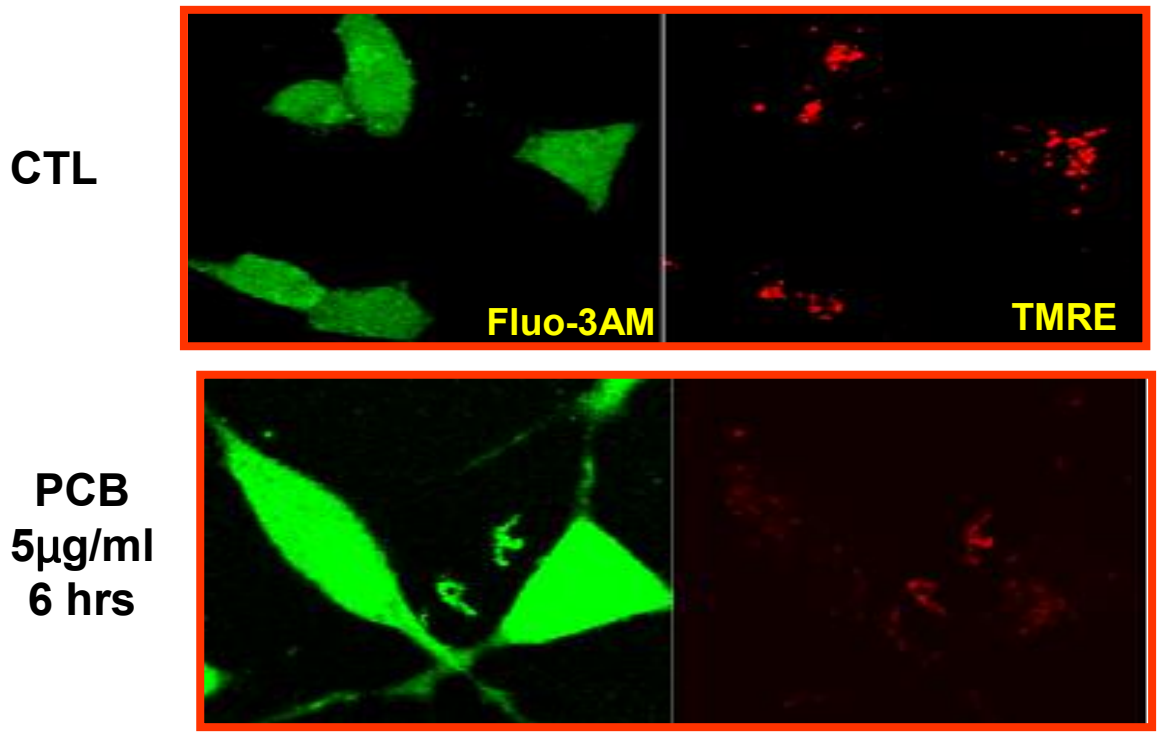


Fig.9: Effect of Aroclor-1254 in mitochondrial oxidative activity after 24hrs exposure SH-SY5Y cells. Neuroblastoma cells stained with FLUO 3AM and TMRE probes in CTL cells and after 6 hrs of treatment with PCB mixture Aroclor-1254 analyzed by confocal microscopy.

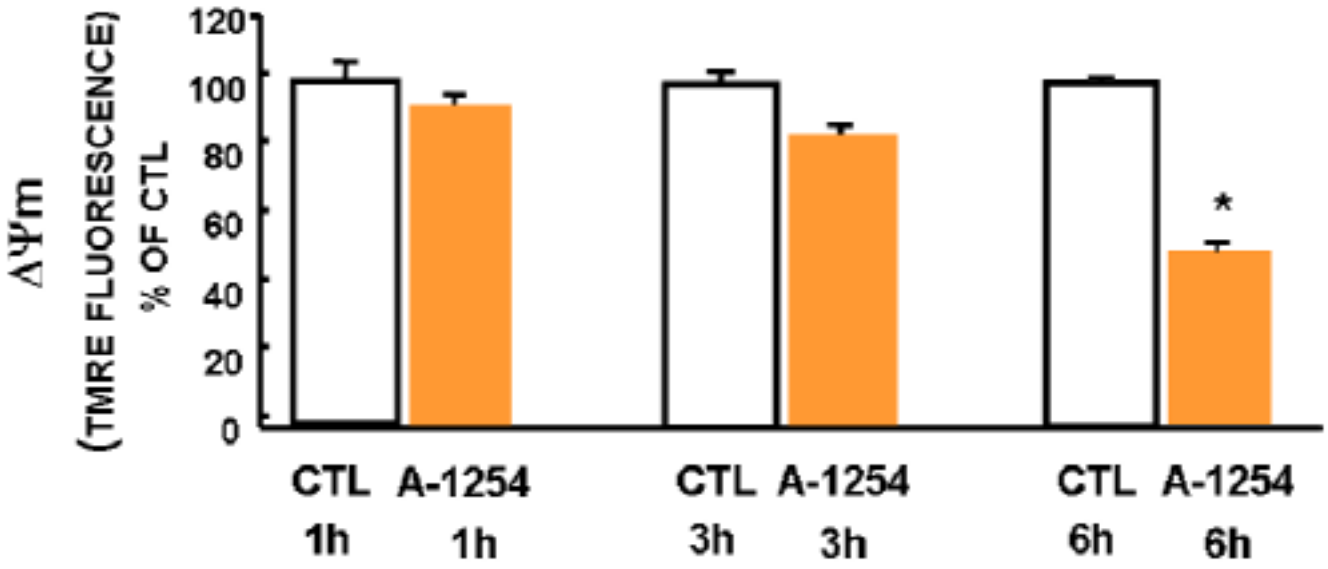


Fig.10: Effect of Aroclor-1254 5 µg/ml on membrane mitochondrial potential in SH-SY5Y cells. TMRE analysis of SH-SY5Y cells exposed to PCB mixture. Cumulative data are expressed as mean 7s.e. of changes in TMRE fluorescence and represent increase over control cells, which was set as 100. The intensity of fluorescence was evaluated in single cells with Meta Morph software analysis. *P<0.05 versus CTL cells (DMSO exposed). Statistical analysis was performed by ANOVA and Newman–Keuls methodology.

The reduction of membrane potential was accompanied by a corresponding reduction in the production of ATP (Fig.11).

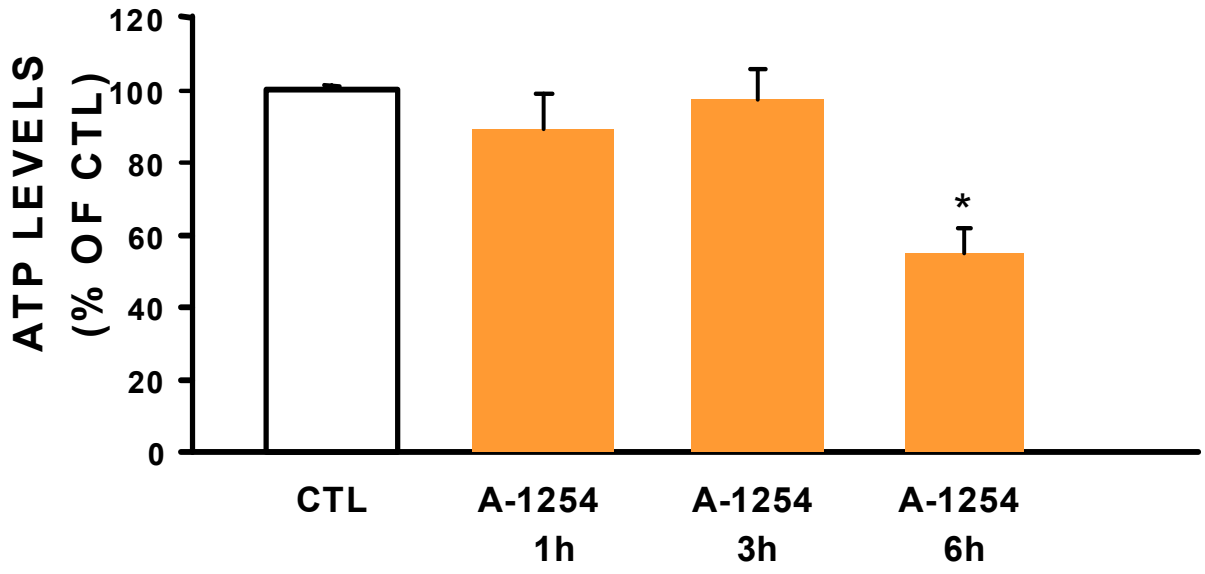


Fig.11: Effect of Aroclor-1254 5 µg/ml on ATP levels in SH-SY5Y cells. Effect of Aroclor 1254 5 µg/ml on ATP content in SH-SY5Y cells. The bar graph represents the ATP content measured after 1-3-6 hrs of treatment. *P < 0.05 vs CTL cells. Each bar represents the mean (±S.E.M.) of 6 different experimental values studied in three independent experimental sessions.

4.3 EFFECT OF PYRUVATE ON PCB-INDUCED MITOCHONDRIAL SUFFERING MITOCHONDRIAL POTENTIAL REDUCTION, DECREASE OF ATP PRODUCTION AND REDUCTION OF ACTIVITY OF COMPLEXES.

The pyruvic acid was administrated to the concentration of 1, 3 and 10mM. These last two concentration were able to significantly prevent the neurotoxic damage induced by PCBs, measured as percentage of cell death (ratio propidium iodide / fluorescein + propidium iodide) (Fig. 12)

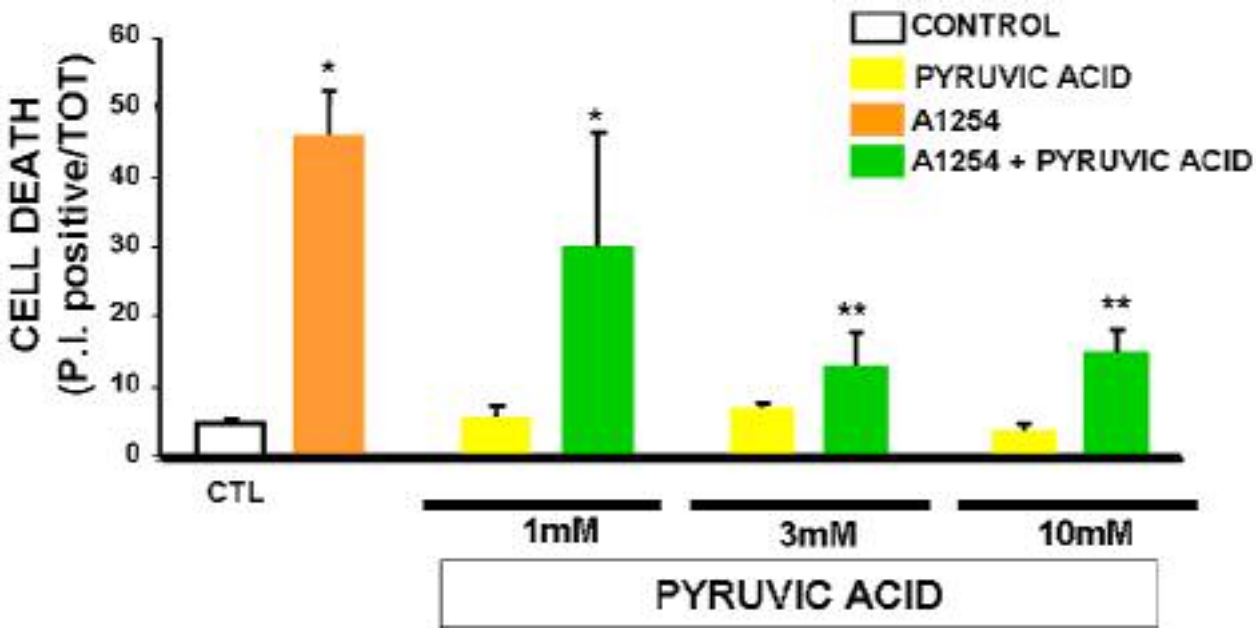


Fig.12. Effect of Pyruvic Acid on cell death induced by 24 hrs of Aroclor-1254 exposure in SH-SY5Y cells. Cell death was measured after 24 hrs of treatment with the PCB mixture Aroclor 1254 5 µg/ml in SH-SY5Y cells. Each bar represents the mean (±S.E.M.) of 15 different experimental values studied in three independent experimental sessions. *$P < 0.05$ vs. CTL cells,**$P < 0.05$ vs. Aroclor 1254 5 µg/ml.

In particular, at the concentration of 3mM, Pyruvic acid prevented the A1254-induced cell injury in neuroblastoma cells (Fig.13).

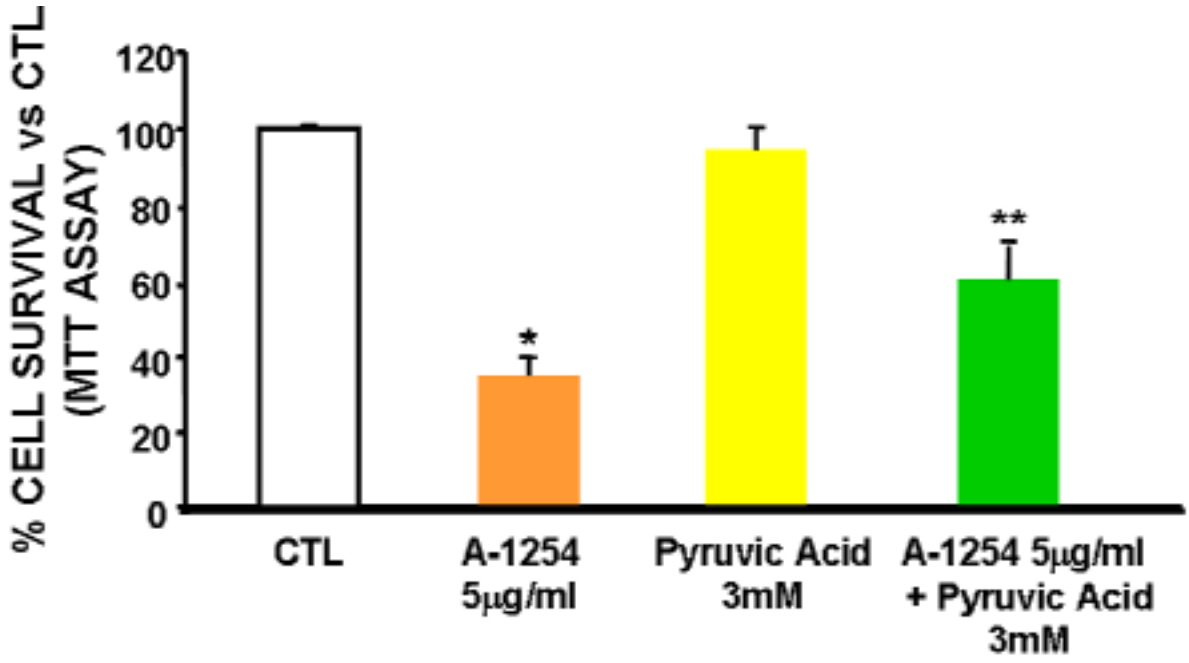


Fig.13: Effect of Pyruvic Acid on Aroclor-1254-Induced Decrease of Mitochondrial Oxidative Activity in SH-SY5Y cells. SH-SY5Y cells were exposed (24 hr) simultaneously to Pyruvic Acid (3mM) and to PCBs mixture A1254 5 µg/ml. Each bar represents the mean 6 SEM (10 separate experiments) of cell viability assessed by MTT assay. *P < 0.05 vs CTL, **P < 0.05 vs A-1254 (5 µg/ml).

These studies seem to suggest even a protective action exerted by pyruvate on mitochondrial suffering. In fact this substance at a concentration of 3 mM was able to significantly prevent the reduction of mitochondrial membrane potential after 6 hrs of treatment (Fig. 14)

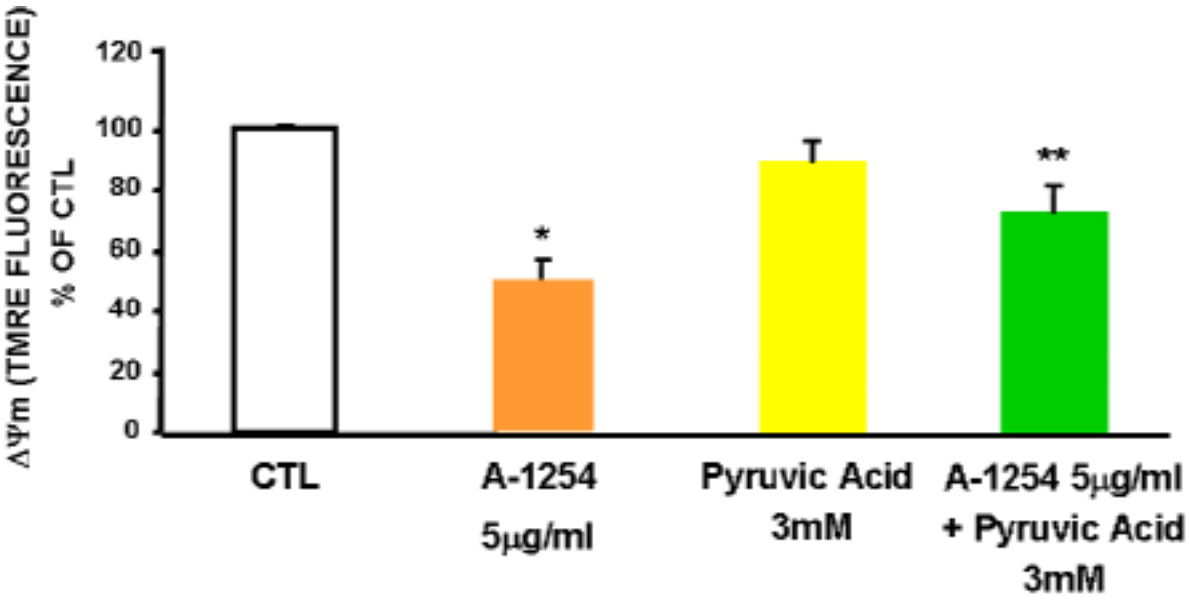


Fig.14: Effect of Pyruvic Acid on Aroclor-1254-induced Decrease of Membrane Mitochondrial Potential (ΔΨm) in SH-SY5Y cells. TMRE analysis of SH-SY5Y cells exposed to Pyruvic Acid 3mM and PCB mixture A1254 5 μg/ml for 6hrs. Cumulative data are expressed as mean ±s.e. of changes in TMRE fluorescence and represent the increase over control cells, which was set as 100. The intensity of fluorescence was evaluated in single cells with Meta Morph software analysis. *P<0.05 versus CTL cells (DMSO exposed), **P<0.05 versus A1254 5 μg/ml. Statistical analysis was performed by ANOVA and Newman–Keuls methodology.

At the same concentration and the same time stimulation, Pyruvic acid was also able to prevent the PCB mediated-ATP reduction (Fig.15)

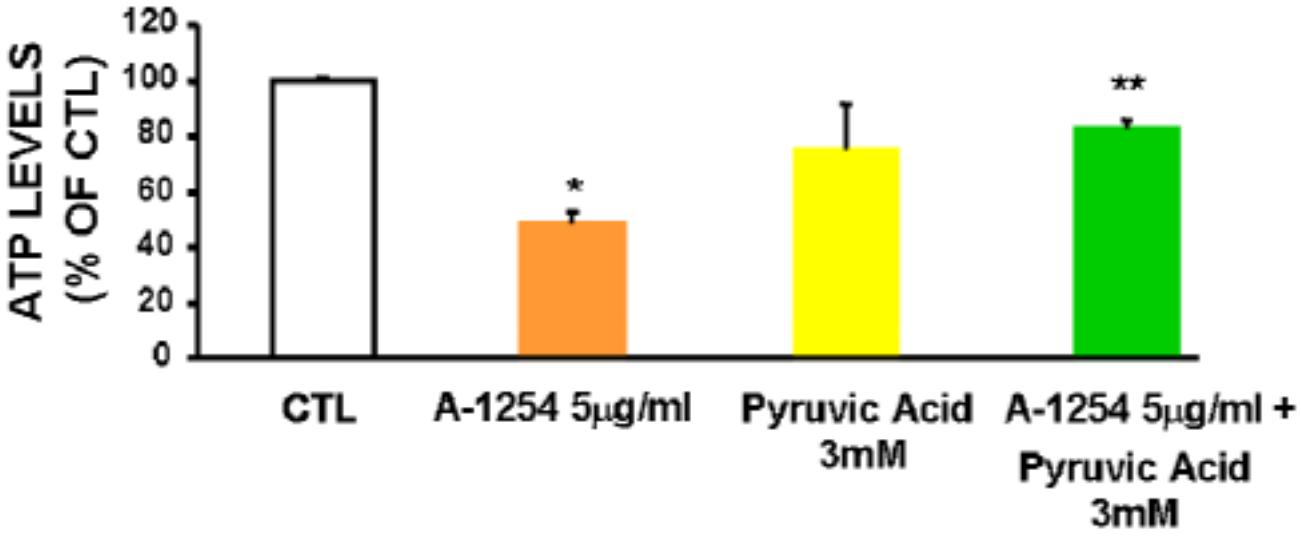


Fig. 15 Effect of Pyruvic acid on Aroclor-1254 –induced decrease of ATP levels in SH-SY5Y cells. Effect of Pyruvic Acid 3mM on ATP content in SH-SY5Y cells exposed to A1254 5 μg/ml for 6hrs. Each bar represents the mean (±S.E.M.) of 6 different experimental values studied in three independent experimental sessions. *P < 0.05 vs CTL cells, P<0.05 versus A1254 5 μg/ml.

The mixture of PCBs Aroclor 1254 (5 μg/ml) administered for 6 hours caused the reduction of mitochondrial enzyme activity of all protein complexes analyzed (complex I, II, IV, V). Pyruvic acid (3mM) was able to completely prevent the reduction of mitochondrial complex activity on each one of the protein complexes (Fig. 16 a, b, c, d). Then, the histochemical staining for the complex V show the impairment of the activity by PCBs and the consequent reversion occurred by pyruvic acid (fig.16 A).

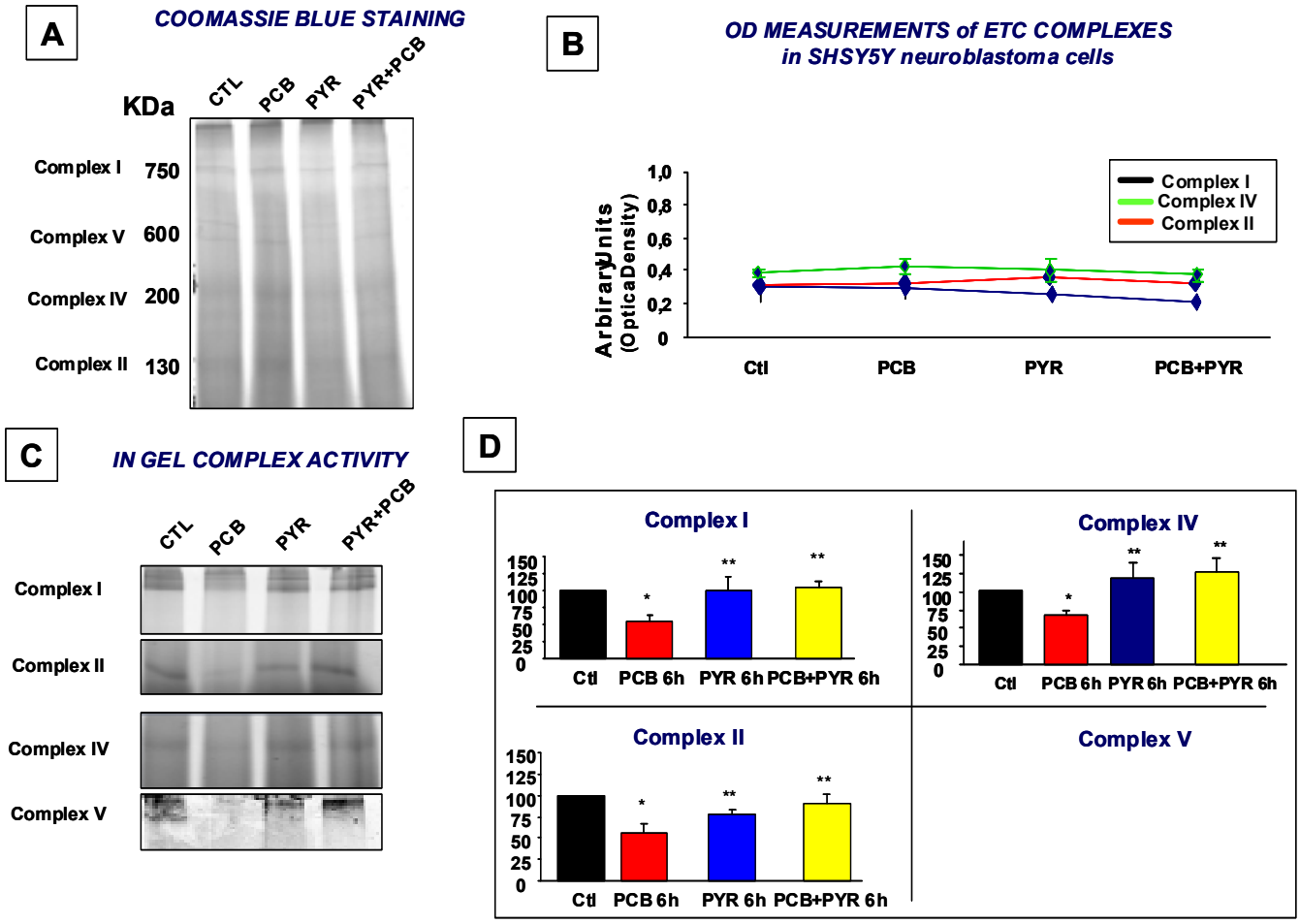


Fig.16: Representative BN-PAGE of dodecylmaltoside-solubilized mitochondrial ETC complexes.

A) Coomassie Blue Staining

Characteristic bands of individual OXPHOS complexes are recognizable in all experimental conditions.

B) Representative density traces for OXPHOS complex bands.

C) Histochemical staining of in gel activity of individual OXPHOS.

D) Quantification of complex activity.

After different treatments, protein extracts were prepared from cultured SHSY5Y cells. ETC complexes were separated by BN-PAGE and stained with Coomassie Blue. The positions and the molecular weights of each complex are indicated on the left. Gel lanes containing ETC complexes were stained for complex I,II,IV and V activities by specific histochemical reactions.
Each lane contained 30µg of mitochondrial protein extracts.
Statistical differences of the data were determined with the Student's t-test for unpaired data. The threshold for statistically significant differences was set at $p<0.05$.
Data are reported as Δ% of increase vs respective control ± SE; *p<0.05 vs ctl; **p<0.05 vs PCBs.

5. DISCUSSION

The results of the present study showed that the exposure for 24 hours to the PCBs mixture, Aroclor 1254 was able to induce a dose-dependent cell death. Furthermore, the inoculation for a shorter time (6 hours) caused a damage of mitochondrial membrane integrity and significantly reduced ATP production. This last event suggest a toxic effects exerted by Aroclor 1254 on mitochondria. That mitochondria could be a target of PCBs toxicity could be contemplated in the basis of the effect of Aroclor 1254 on cell viability evaluated by MTT assay. In fact the reduction of MTT is dependent on the activity of the mitochondrial electron transport chain, impaired formazan production from MTT is usually interpreted as indicative for mitochondrial damage. These findings are in line with those of Hamdy A.A. Aly (2009), who reported that Aroclor 1254 induce citotoxicity and mitochondrial dysfunction in cultured rat hepatocytes, which manifested itself in terms of reduction of MTT viability, reduction of membrane mitochondrial potential, increase of LDH release and inhibited mitochondrial respiratory chain complexes I and III. A further evidence of the mitochondrial damage induced by Aroclor 1254 results by the findings showing that Aroclor induced a decrease of ATP production. In fact 6 hrs of exposure with the Aroclor 1254

simultaneously occurred a reduction of mitochondrial potential and of ATP production.

Several studies had suggested that increased cellular oxidative stress can be the critical underlying mechanism of PCB-mediated cell dysfunction (*Mariussen et al., 2002; Twaroski et al.,2001c*). Thus, Aly et al. (2009) demonstrated that Aroclor 1254 induced ROS production in rat testicular mitochondria that resulted in an impaired spermatogenesis. Since mitochondria are likely the major site of ROS generation (*James et al., 2005; Talbot et al., 2004*). This toxic effect of Aroclor 1254 on neuroblastoma cells mitochondria may be due to the increased lipid peroxidation, in fact lipids play a critical role in maintaining membrane structure and function, and ultimately cellular viability (*Sridhar et al., 2004; Banudevi et al.,2005*). In line with this hypothesis in a previous work we showed that Polychlorinated biphenyls increased the nNOS causing the increase of NO production which can generate reactive nitrogen species (RNS) (*Canzoniero et al.2006*) On the other hand it is well know that mitochondria, which generate ATP through oxidative phosphorylation are among the main targets of ROS (reactive oxygen species) and RNS (wrens nitrogen species). The hypothesis that free radicals overproduction induced by Aroclor-1254 could be responsible for the mitochondrial damage seems to be supported by the results showing that several

antioxidants have been widely studied as therapeutic agents in several neurodegenerative disorders, like pyruvate that possess beneficial effects attributable to its property of neutralizing various ROS, as well as its ability to provide bioenergetic support to the tissue by stimulating glycolysis and/or the citric acid cycle. It also encourages several anaplerotic reactions, replenishing the intermediates of the citric acid cycle and regeneration of glutathione (GSH) from oxidized GSH. It has also been shown to inhibit protein glycation. The treatment with pyruvic acid, a well know scavenger agent, at the concentration of 3mM completely prevented the cellular death Aroclor-1254 inducted and exerted a protective action on mitochondrial suffering. In fact this agent was able to significantly prevent the reduction of mitochondrial membrane potential ($\Delta\Psi m$). It is well known that depolarization and collapse of $\Delta\Psi m$ is one of the early events in the apoptotic cascade. In fact it has been shown that depolarization of $\Delta\Psi m$ can be initiated by opening of mitochondrial permeability transition pores, followed by release of pro-apoptotic factors such as cytochrome c from the mitochondrial inter-membrane space. Pyruvate, a readily oxidized metabolic fuel, could bolster cytosolic energy state (*Mallet and Bunger, 1993; Mallet et al., 2005*), thereby providing energy to maintain cellular functions in the face of oxidative stress (*Nicholls et al., 2003*) and indeed, we found that

pyruvate could maintain ATP production compromised by Aroclor 1254. These findings are in line with Xiaofei Wang et al., (2006) which showed that pyruvate protects mitochondria from oxidative stress in human neuroblastoma SK-N-SH cells.

Another interesting finding emerging from the present study is that, Aroclor 1254 inhibited mitochondrial respiratory chain complexes I, III, IV and V and this inhibition of activity could reflect mitochondrial impairment. Regarding the possible mechanism of this effect it should be underlined that Hamdy et al. (2009) showed that Aroclor 1254 decreased levels of cardiolipin in treated hepatocytes. Cardiolipin is a mitochondrial anionic phospholipid known to confer stability and fluidity to the mitochondrial membrane (*Fariss et al.,2005*). Moreover, cardiolipin also functions as a support for the electron transport chain, since it anchors complexes III and IV, forming a supercomplex (*Hauff and Hatch, 2006*). Therefore the oxidation of cardiolipin induced by Aroclor, could have caused the disruption of the respiratory chain, observed in the present study. In this regard, we observed that pyruvate fully restored the enzymatic activity of each protein complex of mitochondrial chain. This effect can be mainly due to the antioxidant property of pyruvate, and to its capacity to neutralize the lipid peroxidation into the mitochondrial membrane. In fact it has also been reported that the inhibition of respiratory complexes I or III

causes electrons to accumulate within respiratory chain components and these electrons can be added directly to oxygen molecules to produce O_2^- (*St-Pierre et al., 2002; Turrens et al., 1985*). Here, inhibition of complexes I and III induces robust O_2^- generation. Wang et al. (2006) tested the effect of pyruvate on O_2^- generation by submitochondrial particles and showed that Pyruvate not only inhibited O_2^- production under basal conditions but also suppressed O_2^- generation induced by either complex I or complex III inhibition.

In conclusion, our results showed that Aroclor 1254 caused cellular death, mitochondrial potential membrane depolarization and inhibition of mitochondrial respiratory complexes with consequent reduction of ATP production. These effects could be completely prevented by pyruvic acid that maintained ΔΨm, the enzymatic activity of the chain complexes protein under oxidative stress and the normal rate of ATP production. Collectively, these findings indicate that mitochondria are one of the most important and earliest cell targets in Aroclor 1254-mediated toxicity and that PCB induced cell injury that can be prevented by pyruvate. Therefore, antioxidant therapy with pyruvate targeted to mitochondria may constitute apossible strategy to ameliorate the neurotoxic effects exerted by policlorinated biphenyls.

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