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"BIOLOGICAL EFFECTS OF CADMIUM ON CELLS, ORGANS AND TISSUES"

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1 INTRODUCTION

1.1 Cadmium: characteristics and physical-chemical properties

Cadmium is a chemical element with the symbol Cd and atomic number 48. Cadmium is a soft, malleable, ductile, bluish-white bivalent metal; it is chemically similar to the two other metals in group 12 (IUPAC style; group II B), zinc and mercury. Similar to zinc it prefers oxidation state +2 in most of its compounds and similar to mercury it shows a low melting point compared to transition metals. Cadmium and its congeners are not considered transition metals, in that they do not have partly filled d or f electron shells in the elemental or common oxidation states [Cotton, 1999]. Average concentration in the earth's crust is between 0.1 and 0.5 parts per million (ppm). It was discovered simultaneously by Stromeyer and Hermann, both in Germany, as an impurity in zinc carbonate [1].

Cadmium bar (purity 99.999 %)

Cadmium occurs as a minor component in most zinc ores and therefore is a byproduct of zinc production. Cadmium was used for a long time as a pigment and for corrosion resistant plating on steel. Cadmium compounds were used to stabilize plastic. With the exception of its use in nickel-cadmium batteries and cadmium telluride solar panels, the use of cadmium

is generally decreasing in its other applications. These declines have been due to competing technologies, cadmium's toxicity in certain forms and concentration and resulting regulations [2]. Although cadmium is toxic, one enzyme, a carbonic anhydrase with cadmium as reactive center has been discovered.

It is similar in many respects to zinc but forms complex compounds [Holleman et al., 1985]. The most common oxidation state of cadmium is +2, though rare examples of +1 can be found. Cadmium burns in air to form brown amorphous cadmium oxide (CdO). The crystalline form of the same compound is dark red and changes color when heated, similar to zinc oxide. Hydrochloric acid, sulfuric acid and nitric acid dissolve cadmium by forming cadmium chloride ($CdCl_2$) cadmium sulfate ($CdSO_4$) or cadmium nitrate ($Cd(NO_3)_2$).

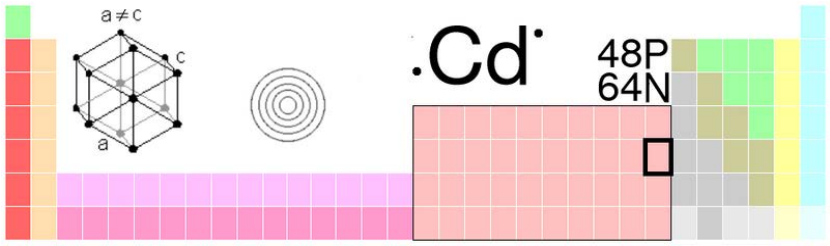
Naturally occurring cadmium is composed of 8 isotopes. For two of them, natural radioactivity was observed, and three others are predicted to be radioactive but their decay is not observed, due to extremely long half-life times. The two natural radioactive isotopes are ^{113}Cd and ^{116}Cd. The other three are ^{106}Cd, ^{108}Cd and ^{114}Cd; only lower limits on their half-life times have been set. At least three isotopes - ^{110}Cd, ^{111}Cd, and ^{112}Cd - are stable. Among the isotopes absent in natural cadmium, the most long-lived are ^{109}Cd and ^{115}Cd. All of the remaining radioactive isotopes have very short

half-lives. This element also has 8 known meta states, with the most stable being ^{113m}Cd, ^{115m}Cd, and ^{117m}Cd.

The known isotopes of cadmium range in atomic mass from 94.950 u (^{95}Cd) to 131.946 u (^{132}Cd). For isotopes lighter than 112 u, the primary decay mode is electron capture and the dominant decay product is element 47 (silver). Heavier isotopes decay mostly through beta emission producing element 49 (indium).

Silver ← **Cadmium** → Indium

Zinc
↓
Cadmium
↓
Mercury



Periodic table of the chemical elements

General properties	
Name, Symbol, Atomic number	cadmium, Cd, 48
Element category	Transition metal
Group, Period, Block	12, 5, d
Density, Mosh Hardness	8650 kg/m^3, 2
Aspect	Bluish-white metal

Miscellanea	
Electronegativity	1.69 (Pauling scale)
Specific heat capacity	26.020 J/(mol*K)
Electrical resistivity	72.7 nΩ*m
Thermal conductivity	96.6 W/(m*K)
1st ionization energy	867.8 kJ/mol
2nd ionization energy	1631.4 kJ/mol
3rd ionization energy	3616 kJ/mol

Atomic properties	
Standard atomic weight	112.411 g/mol
Atomic radius	151 pm
Covalent radius	148 pm
Vn der Waals radius	158 pm
Electron configuration	[Kr]4d^{10} 5s^2
Electrons per shell	2, 8, 18, 18, 2
Oxidation states	2, 1 (milly basic oxide)
Crystal structure	Hexagonal

Most stable isotopes					
iso	**NA**	**TD**	**DM**	**DE**	**DP**
^{108}Cd	0,89%	Cd is stable with 60 neutrons			
^{109}Cd	Syn	462.6 daysni	ε	0,214	^{109}Ag
^{110}Cd	12,49%	Cd is stable with 62 neutrons			
^{111}Cd	12,8%	Cd is stable with 63 neutrons			
^{112}Cd	24,13%	Cd is stable with 64 neutrons			
^{113}Cd	Syn	7.7×10^{15} years	β$^-$	0,316	^{113}In
^{113}Cdm	Syn	14.1 years	β$^-$ IT	0,580 0,264	^{113}In
^{114}Cd	28.73%	Cd is stable with 66 neutrons			
^{116}Cd	7.49%	Cd is stable with 68 neutrons			

Physical properties	
Room temperature status	Solid
Melting point	594.22 K (321.07 °C)
Boiling point	1040 K (767 °C)
Molar volume	13.00×10^{-6} m^3/mol
Heat of vaporization	100 kJ/mol
Heat of fusion	6.192 kJ/mol
Vapor pressure	14.8 Pa a 597 K
Speed of sound	2310 m/s at 293.15 K

Summary table of Cadmium properties

1.2 Cadmium: history

Friedrich Stromeyer

Cadmium (Latin *cadmia*, Greek *καδμεία* meaning "calamine", a cadmium-bearing mixture of minerals, which was named after the Greek mythological character, Κάδμος Cadmus, the founder of Thebes) was discovered simultaneously by Friedrich Stromeyer [Hermann, 1818] and Karl Samuel Leberecht Hermann, both in Germany, as an impurity in zinc carbonate [2]. Stromeyer found the new element as an impurity in zinc carbonate (calamine), and, for 100 years, Germany remained the only important producer of the metal. The metal was named after the Latin word for calamine, since the metal was found in this zinc compound. Stromeyer noted that some impure samples of calamine changed color when heated but pure calamine did not. He was persistent in studying these results and eventually isolated cadmium metal by roasting and reduction of the sulfide. Even though cadmium and its compounds may be toxic in certain forms and concentrations, the British Pharmaceutical Codex from 1907 states that cadmium iodide was used as a medication to treat "enlarged joints, scrofulous glands [Dunglison, 1866], and chilblains". In 1927, the International Conference on Weights and Measures redefined the meter in terms of a red cadmium spectral line (1 m = 1,553,164.13 wavelengths) [Burdun, 1958]. This definition stood unchanged until 1960.

After the industrial scale production of cadmium started in the 1930s and 1940s the major application was the coating of iron and steel to prevent corrosion [2]. In 1944, 62% and in 1956 59% of the cadmium in the United States was used for this purpose [Lansche, 2008]. The second application was red, orange and yellow pigments based on sulfides and selenides of cadmium. In 1956, 24% of the cadmium used within the United States was used for this purpose [Lansche, 2008]. The stabilizing effect of cadmium-containing chemicals (carboxylates such as the laureate and the stearate) on PVC led to a increased use of those compounds in the 1970s and 1980s. The use of cadmium in applications such as pigments, coatings, stabilizers and alloys declined due to environmental and health regulations in the 1980s and 1990s. In 2006, only 7% of total cadmium consumption was used for plating and coating and only 10% was used for pigments [2]. The decrease in consumption in other applications was made up by a growing demand of cadmium in nickel-cadmium batteries, which accounted for 81% of the cadmium consumption in the United States in 2006 [3].

1.3 Cadmium: occurrence and applications

Cadmium-containing ores are rare and are found to occur in small quantities (about 0.1-0.5 mg/kg of the Earth's Crust). However, traces do

naturally occur in phosphate, and have been shown to transmit in food through fertilizer application [Jiao *et al.*, 2004]. Greenockite (CdS),

Greenockite crystals from Tsumeb Mine, Namibia

the only cadmium mineral of importance, is nearly always associated with sphalerite (ZnS). As a consequence, cadmium is produced mainly as a byproduct from mining, smelting, and refining sulfidic ores of zinc, and, to a lesser degree, lead and copper. Small amounts of cadmium, about 10% of consumption, are produced from secondary sources, mainly from dust generated by recycling iron and steel scrap. Production in the United States began in 1907, but it was not until after World War I that cadmium came into wide use [Plachy, 2010; Fthenakis, 2004]. One place where metallic cadmium can be found is the Vilyuy River basin in Siberia [Fleischer, 1980].

In 2009, 86% of all the cadmium is used in batteries, predominantly in rechargeable nickel-cadmium batteries. The European Union banned the use of cadmium in electronics in 2004 with several exceptions but reduced the allowed content of cadmium in electronics to 0.002% [4].

Most of cadmium which is not consumed in battery production is used mainly for cadmium pigments, coatings and plating. Examples of some uses include:

- In electroplating (6% cadmium) [Scoullos *et al.*, 2001] due to the excellent corrosion resistance of cadmium-plated steel components.
- Helium-cadmium lasers are a popular source of blue-ultraviolet laser light used in fluorescence microscopes and various laboratory experiment [5].
- Cadmium is used as a barrier to control neutrons in nuclear fission [Scoullos *et al.*, 2001].
- Cadmium oxide in black and white television phosphors and in the blue and green phosphors for color television picture tubes [Lee *et al.*, 2002].
- Cadmium sulfide (CdS) as a photoconductive surface coating for photocopier drums [Miller *et al.*, 1991].
- In paint pigments, cadmium forms various salts, with CdS being the most common. This sulfide is used as a yellow pigment. Cadmium selenide can be used as red pigment, commonly called *cadmium red*.
- Cadmium is a component of some compound semiconductors.
- In PVC as heat, light, and weathering stabilizers [Scoullos *et al.*, 2001; Jennings, 2005].

1.4 Cadmium: biological role

Cadmium has no known useful role in higher organisms [Hogan, 2010]. A role for cadmium in lower lifeforms has recently been found. A cadmium-dependent carbonic anhydrase has been found in marine diatoms. Cadmium performs the same function as zinc in other anhydrases, but the diatoms live in environments with very low zinc concentrations and thus the biological system has utilized cadmium in place of zinc to perform that function normally carried out by zinc. The discovery was made using X-ray absorption fluorescence spectroscopy (XAFS), and cadmium was characterized by noting the energy of the X-rays that were absorbed [Lane et al. 2000 and 2005].

1.5 Cadmium: human exposure

The general population is exposed to cadmium from multiple sources. Attention is drawn to the following (UNEP, 2008):

- in the non-smoking general population, food accounts for approximately 90 %. The main food commodities that contribute to cadmium exposure are cereals and vegetables.

- cadmium in crops depends on uptake from soils and the rate of uptake is influenced principally by the forms of the element, the soil physico-chemical properties and the plant species.
- meat and fish normally contain lower cadmium contents. Animal offals such as kidney and liver can exhibit high cadmium concentrations, as these are the organs in animals in which cadmium concentrates (UNEP, 2008).

Less than 10 % of total exposure of the non-smoking general population occur due to inhalation of the low concentrations of cadmium in ambient air (Vahter *et al.*, 1991) and through drinking water (Olsson *et al.*, 2002). If present, smoking and occupational exposure may prevail over food as the main sources of cadmium exposure. Smokers have on average twice the body burden of a non-smoker. Workers may accumulate much higher cadmium levels depending on the type of work. The sources of human exposure to cadmium include industry emissions and contamination of different environmental media, i.e. air, water and soil. The latter may also be contaminated by fertilisers. Cadmium contamination of the environment leads to subsequent food contamination, especially in case of cereals and vegetables.

1.5.1 Occupational exposure

Workers may be exposed to cadmium and cadmium compounds in a variety of occupational settings (Table 1).

Alloy production[a]
Battery production[a]
Brazing
Coating
Diamond cutting
Dry colour formulation
Electroplating
Electrical contacts production
Enamelling
Engraving
Glasswork
Laser cutting
Metallizing
Paint production and use
Pesticide production and use
Phosphorus production
Pigment production and use[a]
Plastics production[a]
Plating
Printing
Semiconductor and superconductor production
Sensors production
Smelting and refining[a]
Solar cells production
Soldering
Stabilizer production
Textile printing
Thin film production
Transistors production
Welding[a]

[a]Activities in which risk is highest because atmospheric concentrations of cadmium can be high and because the number of workers employed is relevant (modified from Odone et al., 1983).

Table 1. Occupations in which there is potential exposure to cadmium and cadmium compounds.

The major sources of such exposure are smelting and refining of zinc, lead and copper ores, electroplating, manufacture of cadmium alloys and of pigments and plastic stabilizers, production of nickel-cadmium batteries and welding. Airborne concentrations of cadmium found in occupational settings vary considerably according to the type of industry and to specific working conditions. Cadmium oxide fumes are generated at high temperatures (US Occupational Safety and Health Administration, 1992) and can be absorbed very efficiently through the lung, while deposition and absorption of dust of different cadmium compounds depends on particle size (Alessio *et al.*, 1983; Thun *et al.*, 1991).

1.5.2 Air

Industrial activities are the main sources of cadmium release to the air and emissions from anthropogenic sources have been found to exceed those of natural origin by an order of magnitude (ATSDR, 1999). Cadmium and cadmium compounds have negligible vapour pressures but may exist in air as suspended particulate matter derived from sea spray, industrial emissions, combustion of fossil fuels, or the erosion of soils (Elinder, 1985). In processes that involve extremely high temperatures (e.g., the iron and steel industries), cadmium can volatilize and be emitted as a vapour (Wilber *et al.*, 1992). Total emission to air from natural sources (mainly volcanoes) is estimated at about 150-2,600 tonnes per year. These figures

may be compared to an estimate of total anthropogenic air emission in 1995 of approximately 3,000 tonnes (Nordic Council of Ministers, 2003). The largest source of atmospheric cadmium is non-ferrous metal production, which contributes about 75 % of total anthropogenic cadmium emissions (Pacyna and Pacyna, 2001). In urban areas of the EU, cadmium concentrations in air are in the range between 1 and 10 ng/m3. Atmospheric cadmium is in the form of particulate matter, which may consist of very small particles (<10 μm) if it is produced by combustion processes. The principal chemical species in air is cadmium oxide, although some cadmium salts, such as cadmium chloride, used as stabilizers and pigments in plastics, can enter the air, especially during incineration (IARC, 1993). As a matter of fact traditional municipal solid-waste incinerators may make a significant contribution to the concentration of cadmium in ambient air and to its deposition rates. The rates of emission of cadmium from incinerators in Europe, Canada and the USA ranged from 20 to 2000 $\mu g/m^3$ from the stacks of traditional incinerators and from 10 to 40 $\mu g/m^3$ from advanced incinerators. Cadmium pollutants present in the air may be transported from a hundred to a few thousand kilometres and have a typical atmospheric residence time of about l-10 days before deposition occurs by wet or dry processes (Elinder, 1985; ATSDR, 1999).

1.5.3 Water environment

Cadmium enters the aquatic environment from numerous diffuse and point sources and by different routes.

At the global level, the smelting of nonferrous metal ores has been estimated to be the largest human source of cadmium released into the aquatic environment (Nriagu & Pacyna, 1988). The cadmium content of ore bodies, mine management policies and climatic and geographical conditions all influence the quantities of cadmium released from individual sites. Contamination can arise from entry into aquifers of mine drainage water, wastewater, overflow from tailing ponds and rainwater run-off from mine areas (WHO, 1992a). Other human sources are spent solutions from plating operations and phosphate fertilizers, which are known to contain cadmium. Atmospheric fall-out of cadmium to water courses and marine waters represents the major worldwide source of cadmium in the environment (Nriagu & Pacyna, 1988). Acidification of soils and lakes may result In mobilization of the metal from soils and sediments toward surface and groundwaters (Impens *et al.*, 1989; WHO, 1992b). Other point sources are mining residue dumps, solid-waste deposits and wastewater of both municipal and industrial origin (Muntau & Baudo, 1992). The average cadmium content of seawater is about 5-20 ng/L in open seas, but increased concentrations of 80-250 ng/L have been reported in French and Norwegian coastal zones. Concentrations measured in European rivers

generally vary between 10 and 100 ng/L (OSPAR, 2002).The concentration of cadmium in drinking-water is generally less than 1 µg/L, but it may increase up to 10 µg/L as a result of industrial discharge and leaching from metal or plastic pipes (Friberg et al., 1971).

1.5.4 Soil and plants

The sources of cadmium in soil are nonferrous metal mines and smelters, agricultural application of phosphate fertilizers, use of batteries, PVC stabilizers, pigments and alloys, sewage-sludge landfill, sewage-sludge and solid-waste incineration, and application of municipal sewage sludge to agricultural soil (UNEP, 1984, 1992; WHO, 1992a,b). Atmospheric cadmium deposition onto soil has generally decreased over the last 20 years in Europe. Recent studies have documented that atmospheric emissions do not presently have a significant impact upon the cadmium content of soils (Bak *et al.*, 1997). The cadmium content of fertilisers depends on its concentration in the raw material used for the production. Nevertheless there is currently no EU legislation limiting the maximum level of cadmium in fertilizers but some countries have permanent exceptions to use national guidelines. The concentration of cadmium in soil can vary widely. In non-polluted are as, concentrations are usually below 1 mg/kg (Friberg et al., 1971, 1974), whereas in polluted areas levels of up to 800 mg/kg have been detected (Friberg et al., 1985; 1986b; WHO, 1992b).

Since cadmium is taken up by plants, an increased soil concentration can result in increased levels in food and feeds (UNEP, 2006). However, the concentration of cadmium in soils is not the primary determinant of cadmium in plants. Cadmium is much less mobile in soils than in air and water. Cadmium residues in plants are normally less than 1 mg/kg (Friberg et al., 1986a); however, plants growing in soil contaminated with cadmium may contain significantly higher levels (UNEP, 1984, 1992; WHO, 1992b).

1.5.5 Cigarette smoke

Tobacco plants naturally accumulate relatively high concentrations of cadmium in the leaves. The cadmium content of cigarette tobacco is generally 1-2 µg per cigarette, although the concentrations differ among regions. A smoker who smokes 20 cigarettes per day has an estimated daily uptake of 2-4 µg and accumulates 0.5 mg cadmium in one year (Lewis et al., 1972; UNEP 1984; Friberg et al., 1985; IARC, 1986a; UNEP, 1992; WHO, 1992b).

1.5.6 Food

A number of studies have reviewed cadmium content in a range of different foods. In a collaborative effort under the SCOOP involving 13 EU Member States, cadmium concentrations ranged from non-detected in many foods to

a high mean of 1.20 mg/kg for cephalopods (EC, 2004a). Meat, eggs, fish and milk products generally contain little cadmium (less than 0.01 µg/g wet weight) whereas internal organs, especially liver and kidney, may contain mach more. In general, vegetable products contain more cadmium than animal products. Dietary exposure to cadmium from food sources is determined not only by cadmium levels in foods, but also by consumption patterns. Some of the food items that contain high cadmium levels are rarely consumed by the general European population (e.g. oilseeds and edible offal) and therefore might not be important for overall intake. Other food items with high consumption in the total population or in some sub-populations can be major contributors to the overall cadmium intake even if they contain only low cadmium levels.

1.5.7 Summary of different exposure pathways

A source-pathway model for general cadmium flow is shown in Fig. 1 and a summary of different exposure sources of cadmium is presented in Table 2. Oral exposure from food is clearly the dominating source of overall cadmium exposure for adult non-smokers with a small potential contribution from house dust in particularly contaminated areas. More highly contaminated individual foods can double total dietary exposure from a median of 2.27 in the mean diet up to a maximum of 4.64 µg/kg b.w. per week in simulated extreme diets.

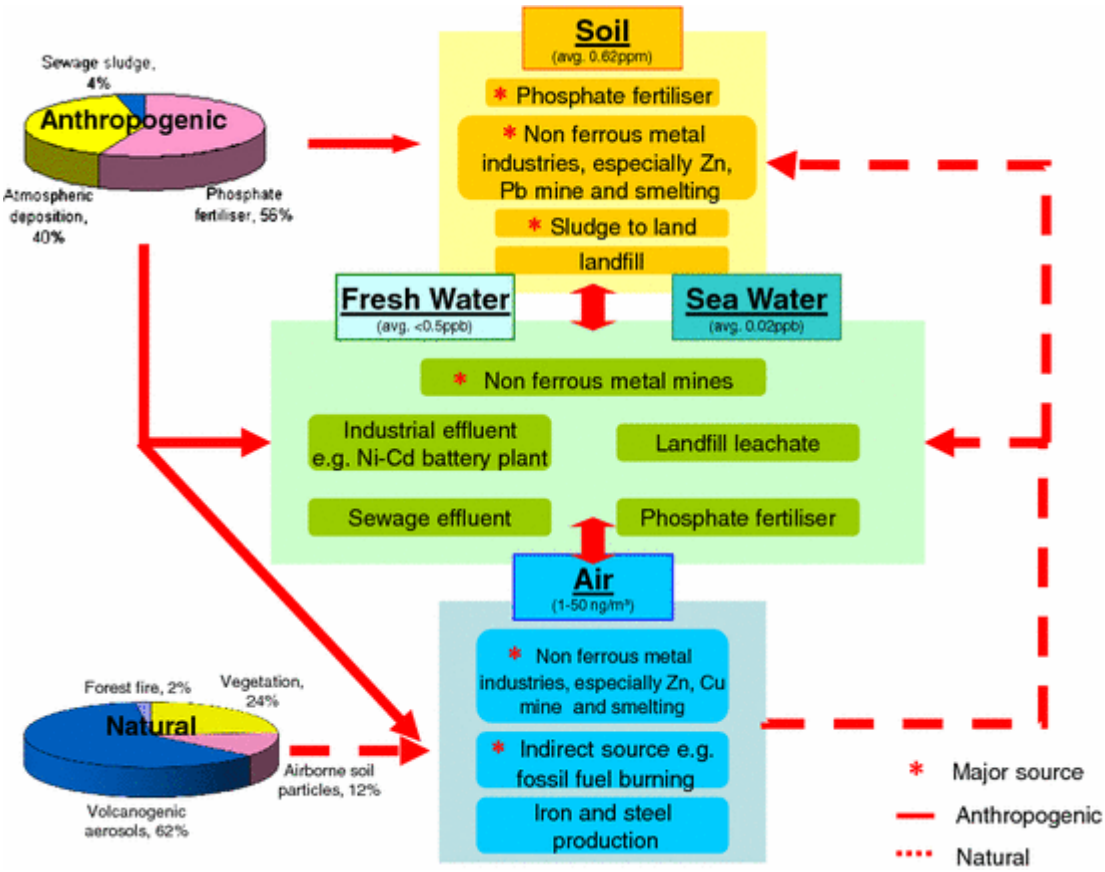


Fig. 1 Source-pathway model for general cadmium flow

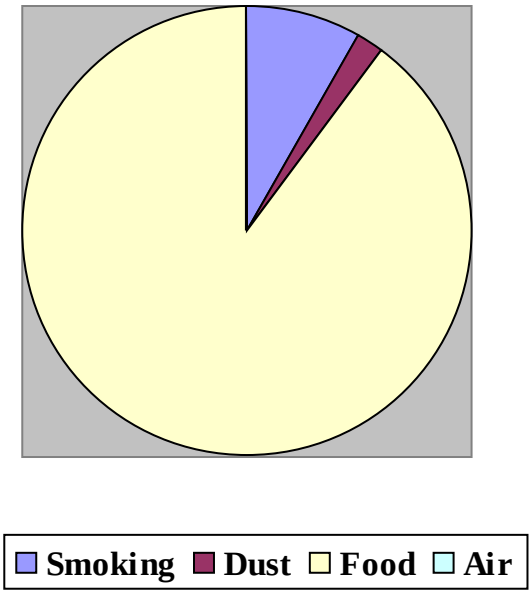
Some vegetarian diets seem to be able to almost triple the mean dietary cadmium exposure. In smokers, the contribution from smoking can increase overall cadmium exposure by 15- 30% when smoking between 20-40 cigarettes a day. This does not take into account the higher pulmonary absorption compared to the gastrointestinal absorption. The potential contribution from different sources for a smoker living in an area with contaminated house dust is illustrated in Figure 2.The special scenario

calculated for children compared to the mean adult scenario showed that children are more exposed.

	Source	Pathway	Range of calculated or reported exposures [µg/kg b.w. per week]	
			Adults	**Children**
Dietary Exposure	Food mean current opinion	Oral	1.89-2.96	2.56-3.46
	Food high current opinion		2.54-3.91	5.49
	Food in industrial areas	Oral	3.3-5.8	4.6
	Extreme diets current opinion	Oral	2.87-4.64	
	Vegetarians	Oral	5.47	
Non-dietary exposure	House dust	Oral	0.076	0.607
	Air	Inhalation	0.0024	0.0033
	Smoking	Inhalation	0.35-0.70	

Table 2. Overview of mean weekly cadmium exposure estimates for the different exposure pathways.

A. Adult smokers



B. Children 0.5-12 years

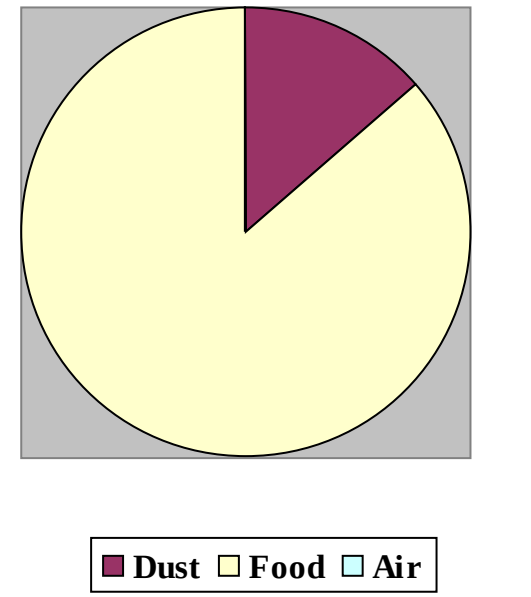


Figure 2. Estimated relative contribution to cadmium exposure of different sources for adult smokers in the presence of contaminated house dust (A) and children in the presence of contaminated house dust (B). The small contribution from air is not visible. The contribution from smoking would increase if the internal dose is considered.

1.6 Cadmium: fate in body

1.6.1 Absorption by route of exposure

Cadmium may enter the body by ingestion, inhalation and, to a very limited extent, by passage through the skin. Although cadmium compounds are relatively poorly absorbed from the gastrointestinal tract (McLellan *et al.*, 1978), the occurrence of systemic toxicity following ingestion (Buckler *et al.*, 1986) indicates that the absorption of cadmium from the gastrointestinal tract does occur and therefore that all cadmium compounds should be considered potentially harmful if ingested. During chronic background exposure, gastrointestinal absorption of cadmium is of the order of 2-8% (McLellan *et al.*, 1978; Friberg & Elinder, 1983; Goyer, 1986). It has been estimated that a European adult absorbs 1.4 - 8 µg of cadmium per day (Lauwerys, 1982). Physiological and nutritional factors may modify this: in people with low body stores of iron the absorption of cadmium may be significantly higher than in subjects with normal iron stores (Flanagan *et al.*, 1978). Cadmium absorption tends to be higher in females than in males. The occurrence of fulminant acute pneumonitis and pulmonary oedema as well as neurotoxic effects following exposure to cadmium-containing dusts and fumes (see e.g. Beton *et al.*, 1966; Barnhart & Rosenstock, 1984) indicates that inhalation of cadmium compounds must be considered serious and potentially fatal (Sittig, 1985; Lenga,

1988). Cadmium concentrations in ambient air are in the order of 0.001-0.005 µg/m3 in rural areas, 0.003-0.05 µg/m3 in urban areas and up to 0.6 µg/m3 near cadmium-emitting sources (Bernard & Lauwerys, 1986a). Much higher concentrations may occur in particular occupational environments. The rate of absorption of cadmium through the lungs is a function of the solubility and surface area of the inhaled particles. 20-30% of the inhaled cadmium is probably retained in the lungs. Therefore, the amount of cadmium retained in the respiratory tract would be 0.005-0.025 µg in rural areas, 0.015-0.250 µg in urban areas and up to about 3 µg per day in areas near cadmium-emitting sources. Cigarette smoking adds considerably to cadmium intake. Friberg *et al.* (1974) estimated a daily intake of 2-4 µg of cadmium from the smoking of one packet of cigarettes per day. Although dermal absorption of cadmium is not likely to be important, skin irritation may be caused by some cadmium compounds (Sittig, 1985; Lenga, 1988). Exposure to many cadmium compounds as well as cadmium dusts and fumes is likely to produce severe corrosive damages to the eye (Lenga, 1988).

1.6.2 Transport

In the blood, cadmium is mainly found in the erythrocytes, where it is mainly bound to metallothionein (MT), a low-molecular weight protein that strongly binds cadmium on its cysteine (sulfur SH) rich groups (Nordberg

et al., 1971a). Cadmium may also bind to other SH-rich low-molecular weight peptides or amino acids such as glutathione and cysteine respectively (Zalups and Ahmad, 2003). MT is also present in blood plasma and serves as the main transport for cadmium into the kidneys because of its low molecular weight (Nordberg and Nordberg, 2000). MTs are small, cysteine rich metal binding proteins which participate in an array of protective stress responses (Lehman-McKeeman and Klaassen, 1987). They likely evolved as a mechanism to regulate zinc levels and distribution within cells. Seven zinc atoms are shared between two clusters (with 3 and 4 binding sites) of the MT monomer, but cadmium can substitute zinc in both clusters. The small size of MT enables the protein to be filtered through the kidney glomerular membrane. It is then reabsorbed into proximal tubular cells (Nordberg *et al.*, 1971b; Foulkes, 1978). Cadmium exposure induces the synthesis of MTs in several tissues (Nordberg *et al.*, 1985). Cd-MT has a longer half-life *in vivo* than Zn-MT. Early work indicated that MT binding decreased the toxicity of cadmium, and the ability of the liver to synthesise MT appeared to be adequate to bind all the accumulated cadmium (Nordberg *et al.*, 1971b; Kotsonis and Klaassen, 1978). Cadmium-induced nephrotoxicity is probably due to unbound cadmium as the ultimate toxicant (Nomiyama and Nomiyama, 1986), as shown in Figure 3. The route of cadmium administration does not appear to affect the MT metabolism in liver and kidney, though inhalation induces

lung MT (Glaser *et al.*, 1986; Hart, 1986) and oral exposure induces intestinal MT (Muller *et al.*, 1986). A schematic presentation of cadmium metabolism and nephrotoxicity is illustrated in figure 3. Cadmium bound to albumin is to a large extent taken up by the liver where the complex is split and only minute amounts reach the kidney proximal tubuli (step l in figure 3). In liver cells, cadmium induces the synthesis of metallothionein (MT). Cadmium is then excreted in bile mainly bound to glutathione (GSH). Redistribution occurs from the liver. Cadmium is either excreted in bile mainly bound to glutathione (GSH), or released into the plasma as Cd-MT. The complex Cd-MT is filtered through the renal glomeruli and then reabsorbed by proximal tubular cells until the critical concentration is reached. The critical concentration is represented by the cadmium burden at which tubular cells are no longer able to synthesise enough MT to neutralise free Cd^{2+} produced by lysosomal degradation of Cd-MT. When the critical concentration is exceeded, tubular damage results in increased enzymuria, low molecular weight proteinuria, and increased excretion of cadmium either as Cd-MT or, probably, also as Cd^{2+}.

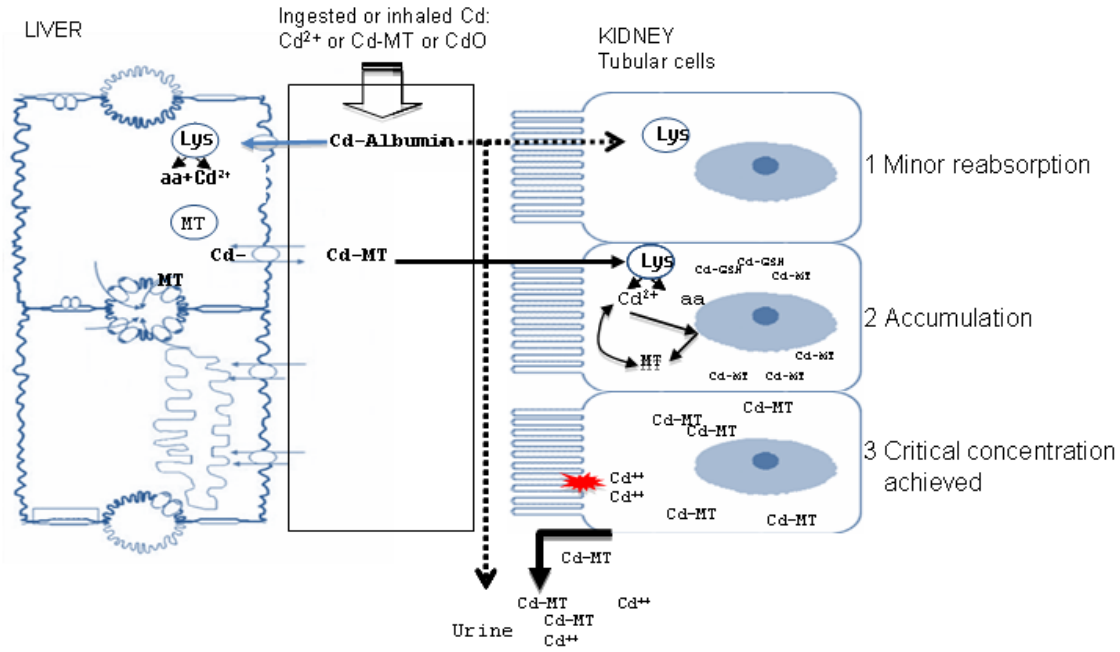


Figure 3. Cadmium metabolism and nephrotoxicity. Upon chronic exposure, cadmium nephrotoxicity depends on the imbalance between the liver ability to synthesise MT carrying cadmium to the kidney and the ability of the latter to synthesise the renal counterpart of MT necessary to neutralise Cd2+ delivered from lysosomal degradation of the Cd-MT complex.

1.6.3 Distribution

Distribution of cadmium in animals after oral exposure shows the highest accumulation in the liver and kidneys (Kotsonis and Klaassen, 1978). Liver and kidney cadmium concentrations are comparable after short-term exposure (Andersen *et al.*, 1988), but the kidney concentration exceeds the liver concentration following prolonged exposure, except at very high exposures (Bernard *et al.*, 1990). Zinc and calcium deficiencies may also result in an increased accumulation of cadmium in the intestinal wall, liver and kidney (Foulkes and Voner, 1981; Reeves and Chaney, 2008). Non-

occupationally exposed people are exposed to cadmium primarily through the diet and either active or passive tobacco smoke. Cadmium can be detected in virtually all tissues in adults from industrialised countries, with the greatest concentrations in the liver and kidney (Sumino *et al.*, 1975; Chung *et al.*, 1986). Average cadmium concentrations in the kidney are near zero at birth, and rise almost linearly with age to a peak (typically around 40-50 mg/kg wet weight) between ages 50 and 60, after which kidney concentrations decline. Liver cadmium levels also begin near zero at birth, increase to typical values of l-2 mg/kg wet weight by age 20-25, then increase only slightly (Sumino *et al.*, 1975; Lauwerys *et al.*, 1984; Chung *et al.*, 1986). In humans subjected to normal low-level exposures, approximately 50% of the total cadmium body burden at autopsy is found in the kidney and 15 % in the liver, and only a relatively small part is stored in bone (Christoffersen *et al.*, 1988; Nordberg *et al.*, 2007). In several studies, including both smoking and non-smoking women, the cadmium concentration was approximately half as high in cord blood as in maternal blood (Lauwerys *et al.*, 1978; Kuhnert *et al.*, 1982; Truska *et al.*, 1989). Accumulation of cadmium in the placenta at levels about 10 times higher than maternal blood cadmium concentration has been observed in studies of women in Europe, e. g. (Roels *et al.*, 1978) and the United States (Kuhnert *et al.*, 1982). The thyroid, the pancreas and the salivary glands also accumulate significant amounts of cadmium.

1.6.4 Metabolism

There is no evidence that the divalent cadmium cation undergoes biotransformation in man.

1.6.5 Excretion

Most of the inhaled cadmium that is transported to the gut via mucociliary clearance is not absorbed from the gastrointestinal tract (Moore *et al.*, 1973; Rusch *et al.*, 1986). Absorbed cadmium is excreted very slowly, with urinary and faecal excretion being approximately equal. The amount excreted daily represents only about 0.007-0.009% of the total body burden (Kjellström and Nordberg, 1978; Nordberg *et al.*, 1985). Low excretion rates of cadmium lead to efficient retention in the body. The biological half-time in kidneys was estimated to be 12-20 years (Elinder et al., 1976; Kjellström & Nordberg, 1978; Roels et al., 1981) and that in the liver somewhat shorter (Kjellström & Nordberg, 1978).

1.7 Cadmium: toxicity

1.7.1 History

The earliest observations of toxic effects from cadmium exposure are from clinical medicine. Sovet (1858) reported on acute gastrointestinal

symptoms as well as delayed respiratory symptoms among persons using cadmium carbonate powder as a polishing agent causing both inhalation and oral intake of this cadmium compound. Early toxicological contributions were those by Alsberg and Schwartze (1919) and by Schwartze and Alsberg (1923) on the pharmacology of cadmium. They were based on animal experiments and reported various clinical signs and morphological changes in organs of a variety of vertebrates including birds and dogs. Acute gastrointestinal effects with vomiting and diarrhea in persons consuming Cd contaminated food and drink were reported in the 1940's (US Publ Health Serv, 1942). Nicaud et al. (1942) observed a systemic effect, osteomalacia, in a few Cd-workers. Friberg (1948) presented the first report on the characteristic combination of proteinuria and emphysema in chronic cadmium poisoning occurring among Cd workers. Itai-itai disease (Japanese for Ouch-Ouch disease) is a bone disease with fractures and severe pain that occurred after World War II in Fuchu, Toyama prefecture, Japan (Hagino, 1957) (figure 4).

Dr. Hagino was a local practitioner who initiated studies concerning this disease. X-ray findings included Milkman's pseudofractures (Looser´s zones) in the long bones, changes characteristic of osteomalacia. There were also decalcification and fractures of other bones including compression fractures of the spine. In the area where the disease occurred,

women had the habit of screening out sunlight by wearing typical dress with wide hats, thus limiting dermal synthesis of active vitamin D.

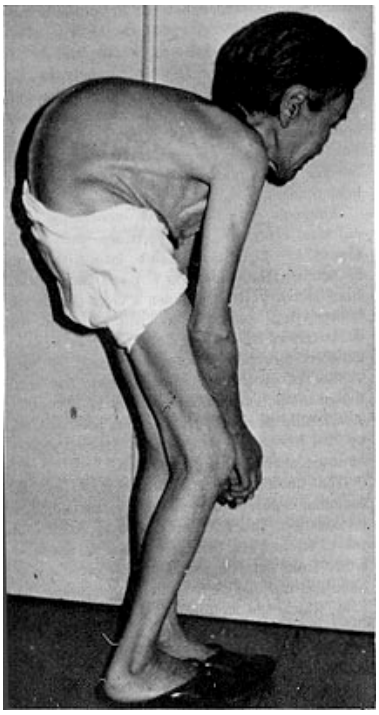


Figure 4. Itai-Itai disease induced by long-term Cadmium-exposure.

Biochemical findings were characteristic of osteomalacia with increased serum levels of alkaline phosphatase and decreases in calcium and phosphate. Anemia and gastrointestinal and renal dysfunction were other less prominent findings [Ishizaki, 1969, Murata et al., 1970]. In these cases, elevated levels of Cd in urine were found [Ishizaki, 1969] and in 1968 the disease was declared by the Japanese Government to be a disease related to environmental pollution. Cadmium, released from a mine in the mountains was transported by the Jinzu River into the plain, where the contaminated water was used for irrigation of rice fields (figure 5). The rice plant takes

up Cd from the soil and consumption of contaminated rice is the main route of exposure for the general population.

Figure 5. Acid mine drainage of heavy metals including cadmium, lead, andarsenic threatens waterways with toxic contamination.

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1.7.2 Health effects

1.7.2.1 Kidney

There is now a consensus among scientists to say that in chronic Cd poisoning the kidney, which is the main storage organ of Cd, is also the critical target organ, *i.e*. the first organ to display signs of toxicity [Berbard A., 2008]. The earliest manifestation of Cd-induced renal damage considered as critical consists in an increased urinary excretion of microproteins (molecular weight <40 kD). Among these proteins, β2-microglobulin, retinol-binding protein and alpha1-microglobulin

have been the most validated for the routine screening of tubular proteinuria. The increased loss of these proteins in urine is a reflection of the decreased tubular reabsorption capacity. A modest increase in the urinary excretion of these proteins, as found at the early stage of Cd nephropathy, is unlikely to compromise the renal function [Berbard A., 2008]. Such a small increase might even be reversible after removal from Cd exposure. By contrast, when the urinary excretion of these proteins is increased by more than one order of magnitude, tubular dysfunction caused by Cd becomes irreversible and may be associated with a lower glomerular filtration rate (GFR) and an accelerated decline of the GFR with ageing [Bernard A., 2008]. The disturbances of calcium and phosphate metabolism accompanying Cd nephropathy may lead to bone demineralization, the formation of kidney stones and bone fractures. Prospective studies among inhabitants living in Cd-polluted areas in Japan have shown that the development of Cd-induced proteinuria is predictive of an increased mortality by heart failure, cerebral infarction, nephritis and nephrosis[14]. There is some epidemiological evidence that diabetics are more susceptible to the nephrotoxic action of Cd, a finding consistent with animal studies [Bernard A., 2008].

1.7.2.2 Bone

Although first reported in French workers by Nicaud *et al.* [1942], toxic effects of Cd on the bones really became evident with the outbreak of the Itai-Itai disease in the Cd-polluted area of Toyama, Japan, after World War II. Itai-Itai disease patients presented, indeed, a severe osteomalacia accompanied with multiple bone fractures and renal dysfunction[9]. They complained of pain in the back and in the extremities, difficulties in walking and pain on bone pressure (hence the name Itai-Itai meaning Ouch-Ouch in Japanese). Recent studies in China have confirmed the bone toxicity of Cd. Nordberg *et al*. [2002] have found decreased bone mineral density in Chinese farmers exposed to Cd from contaminated rice for more than 20 yr. The bone mass density was decreased in postmenopausal women with elevated Cd in urine or blood as well as among men with elevated Cd in blood. Bone lesions have been regarded for long as late manifestations of intoxication, occurring only after relatively high exposures in the industry or environment. Effects on the bone, especially at high exposure, are largely the consequence of Cd nephropathy, resulting in an altered vitamin D metabolism and a urinary waste of calcium and phosphate.

1.7.2.3 Others (respiratory system, liver, cardiovascular system , reproductive system and embryo, central nervous system, retina)

Depending on the dose, route and duration of exposure, Cd can damage various other organs and systems including lung, liver, cardiovascular and reproductive systems, embryo, placenta, central nervous system and retina. With regard to the respiratory system, experimental studies have shown that cadmium-containing aerosols induce destruction of Type I epithelial cells with pulmonary oedema, followed by a reparative process. The earliest stages of Cd-induced lung injury primarily involve the disruption of the alveolar septum and the leakage of fluid and solutes into the alveoli [Prozialeck W.C., 2006]. However, these early stages of injury are soon followed by the influx and activation of leukocytes and the release of inflammatory mediators that facilitate the inflammatory processes responsible for subsequent pulmonary injury and fibrosis [Prozialeck W.C., 2006].

With low level oral or respiratory exposure, liver is the first site where Cd is initially distributed; it can bind to glutathione and/or induce the synthesis of the Cd-binding protein metallothionein, which are both thought to serve as intracellular lines of defense against Cd toxicity [Prozialeck W.C., 2006]. Under these conditions, the liver is usually not injured. By contrast, with the acute, higher levels of exposure that are often used in animal studies, the liver rapidly accumulates high

levels of Cd that overcome these defense mechanisms and the liver becomes one of the primary sites of injury. Under these conditions, changes in hepatic vascular endothelial cells are evident after as little as 2–3 h of exposure, and signs of parenchymal necrosis and/or apoptosis develop several hours later [Prozialeck W.C., 2006].With regard to the cardiovascular system, exposure to Cd has been associated with a wide variety of cardiovascular pathologies including hemorrhagic injury, atherosclerosis, hypertension and cardiomyopathy [Prozialeck W.C., 2006]. Cadmium may also cause severe damage to embryos and the reproductive organs in adults including the ovary and testes, which are sensitive to Cd toxicity (for an extensive review, see Thompson and Bannigan, 2008 “Cadmium: Toxic effects on the reproductive system and the embryo”). The toxic effects of Cd in the central nervous system (CNS) are still poorly understood. As a matter of fact the anatomical structure of the blood brain barrier (BBB) in the newborn and in the adult is analogue; nevertheless it has been widely demonstrated that BBB in the newborn has different functional characteristic. Cd, such as other compounds, could pass the BBB exploiting the natural transporters of the essentials metals (Bressler et a., 2007). Furthermore some papers hypothesized that several diseases including Parkinson disease (Dhillon et al., 2008), as well as malformations (Paniagua-Castro et al., 2008) such as spina bifida (Kalter, 1985) and forelimb

ectrodactyly (Schreiner et al., 2009) might be related to Cd exposure. Concerning the role of Cd in the etiopathogenesis of sporadic and familiar amyotrophic lateral sclerosis (ALS), some data identify it as a candidate risk factor (Huang et al., 2006; Bar-Sela et al., 2001; Vinceti et al., 1997); nevertheless more studies are needed to provide a definitive answer concerning the cause-effect relationship between Cd and ALS. Moreover several studies indicate that children of women exposed to Cd during pregnancy show lower motor and perceptual abilities, and that high Cd body burden in children is also related to impaired intelligence, lowered school achievement and nonadaptive classroom behavior (Thatcher et al., 1982; Bonithon-Kopp et al., 1986). The distribution and deleterious effects of Cd in retinal tissues have not yet been extensively studied. Dysfunction of the retinal pigment epithelium (RPE) underlies numerous retinal diseases and has been implicated in age-related macular degeneration (AMD), a leading cause of blindness. Recently, Erie et al. (2005) demonstrated that cadmium is present in the retinal pigment epithelium/choroid at levels higher than in the blood or ocular fluids and that cadmium levels in the retinal tissues were approximately double in smokers compared to nonsmokers. In addition, higher urinary cadmium levels, indicating a higher total body burden of cadmium, were found in smokers who had AMD compared to smokers who did not have AMD [Erie et al., 2007].

These findings raised the possibility that cadmium exposure might play a role in tobacco-related AMD. Moreover, rececently, it was demonstrated that human retinal tissues accumulate Cd during aging [Wills et al., 2008]. Among its numerous cellular toxic effects, cadmium is a potent inflammatory agent and increases oxidative stress. Oxidative stress and inflammation have both been linked to AMD [Hollyfield et al., 2008]. Gender might also influence cadmium absorption. Satarug et al. (2004) reported that women who were non-smokers had nearly the same cadmium body burden as men who smoked an average of nine cigarettes per day for nearly 10 years.

1.7.2.4 Cadmium and cancer

International Agency for Research on Cancer (IARC,1993) classified cadmium as a human carcinogen (group I) on the basis of sufficient evidence for carcinogenicity in both humans and experimental animals, while the European Commission has classified some cadmium compounds as possibly carcinogenic (Carcinogen Category 2; Annex 1 to the Directive 67/548/EEC). IARC's evaluation was largely based on studies on occupationally exposed workers, but study populations were rather small, data on historical exposure to cadmium were sparse and concomitant exposure may have confounded the results as noted in subsequent studies [Järup et al., 2009]. Strong evidence, based on

experimental studies, exists to support the carcinogenic potential of Cd. Cell transformation, a routinely employed diagnostic in vitro test for the carcinogenic potential of chemicals, has been employed in several studies not only to demonstrate the carcinogenic potential of Cd but also to gain insight regarding the mechanisms potentially underlying Cd carcinogenesis [Järup et al., 2009]. Following various routes of exposure to Cd, experimental animals produce tumors of multiple organs. In addition, injection of Cd containing compounds in animals resulted in local tumors, typically sarcomas, at the site of injections. Epidemiological evidence is also available to document the carcinogenic potential of Cd. Although, the evidence is not very strong, cancers of the prostate, kidney, and pancreas have been reported in populations who are exposed to Cd [Järup et al., 2009]. It is, however, worth mentioning that, like any other epidemiological study, the results of studies involving Cd carcinogenesis should be interpreted with caution primarily because of confounding factors such as coexposure to other toxic chemicals and life style factors, for example, cigarette smoking. Despite such limitations, a causal relationship has been observed between Cd exposure and the occurrence of lung cancer in human and the administration of compounds containing Cd have resulted in tumors of multiple organs/tissues in experimental animals prompting the International Agency for Research on Cancer (IARC) to

finally classify Cd as a human carcinogen in 1993 [IARC, 1993]. More recently, cadmium was proposed as a potent metalloestrogen [Järup et al., 2009]. The metal was shown to increase estrogen receptor alpha-mediated cell proliferation [Järup et al., 2009].; and to possess mutagenic properties . Although some in vitro estrogenicity assays showed no activity, environmentally relevant doses of cadmium induced several well-characterized estrogenic responses in vivo, including increased uterine weight, hyperplasia and hypertrophy of the endometrial lining, induction of uterine progesterone receptor and complement C3 gene expression in animals [Järup et al., 2009]. In addition, after in utero exposure, cadmium affected mammary gland development and early onset of puberty in female offspring — both prototypical endocrine disruptor-like responses [Järup et al., 2009]. Furthermore a possible effect of cadmium on breast cancer risk was assessed in a case–control study. Women in the highest quartile of creatinine adjusted urinary cadmium levels had twice the breast cancer risk of those in the lowest quartile after adjustment for established risk factors, and there was a statistically significant increase in risk with increasing cadmium level [Järup et al., 2009]. Increased cadmium concentrations in breast adipose tissue samples from breast cancer patients, and a suggestive correlation of cadmium with estrogen receptor levels has been also demonstrated [Järup et al., 2009]..

1.7.3 Molecular aspects

1.7.3.1 Inhibition of DNA repair

Cadmium is not directly genotoxic, it is non-mutagenic in bacteria and weakly mutagenic in mammalian cells with contrasting evidence. Two mechanisms play an important role in cadmium genotoxicity: 1) induction of reactive oxygen species (ROS) and 2) inhibition of DNA repair. At high cytotoxic doses cadmium induces DNA single-strand breaks and genome instability in several types of mammalian cells likely due to oxidative stress. At sub-cytotoxic concentrations cadmium is also harmful because it can sensitize cells to external agents by inhibiting DNA repair. By this mechanism cadmium can enhance the mutagenicity induced by other DNA damaging agents. The precise molecular mechanism involved in the inhibition of DNA repair by cadmium has not been clearly identified. Cadmium does not directly modify DNA but its capacity to inhibit DNA repair enzymes may contribute to its genotoxic effects.

1.7.3.2 Effects on gene expression

Cadmium affects both gene transcription and translation. Cadmium regulates the internal cell concentration of calcium and plays the role of

an alternative signalling molecule controlling various transduction pathways. Moreover, cadmium may interfere with calcium homeostasis by its ability to modulate extracellular calcium sensing receptors (CaSR). In this way cadmium may profoundly affect the function of cells expressing CaSR such as kidney cells that are involved in calcium homeostasis. A recent study on mice showed that cadmium introduced morphological changes and reduced the level of calcium in the mammary gland and decreased beta-casein gene expression, suggesting that cadmium can disturb the function of the lactating mammary gland, which may impair the development of the suckling offspring. Cadmium is not a redox-active metal and cannot itself direct Fenton type-reactions but it induces the production of ROS by indirect processes, a decrease of cellular antioxidants and exhalation of ROS by mitochondria. Cadmium by perturbing the redox homeostasis impacts a large set of transcription factors characterized by reactive cysteines. Among these, MTF1 which is the inducer of MT. Moreover, by inducing oxidative modification of proteins cadmium can also target these proteins to degradation. By modulation of gene expression and signal transduction cadmium may affect cell proliferation, differentiation, apoptosis and other cellular activities. The perturbation of these processes may all contribute to carcinogenicity.

1.7.3.3 Endocrine functions

The concomitant manifestations of proximal renal tubular dysfunction and anaemia with erythropoietin (Epo) deficiency observed in chronic cadmium intoxication, such as Itai-itai disease, suggest a close local correlation between the cadmium-targeted tubular cells and Epo-producing cells in the kidney. Recent studies show that cadmium has a strong inhibitory effect on Epo expression both *in vitro* and *in vivo*. It is well known that moderate to high dose exposure to cadmium (1 mg/kg for 5 days/week for 6 weeks by *i.p.*) affects steroid synthesis in reproductive organs in female rats. It has been shown that low dose cadmium exposure has potent oestrogen- and androgen-like activities *in vivo* and *in vitro*, by directly binding to oestrogen and androgen receptors. Cadmium, like oestradiol, can cause rapid activation of ERK1/2 and AKT. However, the precise mechanisms underlying the effects of cadmium as an endocrine disruptor remain to be elucidated.

1.8 Notes

Product recalls. In May 2006, a sale of the seats from Arsenal F.C.'s old stadium, Highbury in London, England was cancelled after the seats were discovered to contain trace amounts of Cadmium [6]. Reports of high levels of cadmium use in children's jewelry in 2010 led to a US Consumer

Product Safety Commission investigation. Twelve percent of the 103 items tested from New York, Ohio, Texas and California contained at least 10 percent cadmium, with a single item test claimed to be 91 percent cadmium [7]. The CPSC issued specific recall notices for cadmium content applying to jewelry sold by Claire's [8] and Wal-Mart [9] stores. In June 2010 McDonald's voluntarily recalled more than 12 million promotional “Shrek Forever After 3D” Collectable Drinking Glasses due to concerns over cadmium levels in paint pigments used on the glassware [Neuman, 2010]. The glasses were manufactured by ARC International, of Millville, NJ, USA [10].

1.9 Aim of the study

Even though Cd represents a major environmental health problem, the specific mechanisms by which it produces its adverse effects have yet to be fully elucidated. Studies to address this issue have shown that Cd can cause a variety of biochemical, metabolic and cytotoxic effects. However, in most cases, the relationships between these effects and the specific toxic actions of Cd in various target cells, tissues and organs have not been firmly established.

This study focused on the role of cadmium on human cells, tissues and organs with special attention to morphology, cell proliferation, apoptosis,

differentiation-related gene expression, and angiogenic potential. This research project was subsidized by the Italian National Institute of Health (Istituto Superiore di Sanità – ISS) and the University of Florence (Italy); in particular, our scientific group belonged to the Strategic Project “The gender medicine as a strategic target for public healt: the suitability of the cure for the care of woman health”, Project 3: “Endocrin interferents in occupational environments and woman healt” (Progetto strategico: “La Medicina di genere come obiettivo strategico per la sanità pubblica: l'appropriatezza della cura per la tutela della salute della donna”, Progetto 3: “Interferenti endocrini negli ambienti di lavoro e salute della donna”)**.** For that reason our study focused principally on the biological effects of cadmium on cells, tissues and organs involved in female and foetus health. Hence experimental models adopted were: normal and tumour human breast cell lines (MCF-7 and MCF-10A respectively), primary human neuroblast cell line from foetal olfactory neuroepithelium (FNC-B4), explants of human foetal spinal cord, and, more recently, primary human neurons from foetal basal nuclei and primary (murine) retinal ganglion neurons.

2 MATERIALS AND METHODS

2.1 Cadmium and normal/tumour human breast cell lines (MCF-7 and MCF-10A respectively)

MCF-7 (acronym of Michigan Cancer Foundation **-** **7**, referring to the institute in Detroit where the cell line was established in 1973) is a human breast adenocarcinoma cell line isolated in 1970 from a 69-year-old Caucasian woman. MCF-7 cells are useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the differentiated mammary epithelium. These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line.

The MCF 10A cell line is a non-tumorigenic epithelial cell line. These cells were derived from the mammary tissue of a cystic fibrosis patient and have normal mammary epithelial cell morphology. The line was produced by long term culture in serum free medium with low Ca++ concentration. MCF 10A was derived from adherent cells in the population. Similar to normal human breast epithelial cells, at confluence the MCF-10A cells form dome structures in tissue culture plates; in addition they have shown no signs of terminal differentiation or senescence. MCF-10A cells are frequently used as a normal control in breast cancer studies.

2.1.1 Cell culture and treatments

The MCF-7 and MCF-10A cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA).

MCF-7 were cultured in Eagle's MEM, supplemented with 10% FBS, 1% penicillin/streptomycin, insulin (10ug/mL) and sodium pyruvate (1mM).

MCF-10A were cultured in Ham's F-12 medium and DMEM (1:1) supplemented with 5% FBS, 1% penicillin/streptomycin, insulin (10ug/mL), EGF (20ng/mL), cholera enterotoxin (100 ng/mL), hydrocortisol (0.5 mg/mL).

Materials for cell culture are bought from Invitrogen Co., Carlsbad, CA, USA.

Both MCF-7 and MCF-10A were switched, 24 h before the treatments, from complete culture medium to DMEM serum-free medium; then, at the beginning of each experiment, the medium was replaced with DMEM without phenol red and serum-free medium containing different concentrations of cadmium chloride ($CdCl_2$; Sigma-Aldrich, St. Louis, MO, USA). Treatments with and without $CdCl_2$ are carried out for 48 h.

2.1.2 Histological staining and morphologic analysis

For the study of cell morphology, both the cell lines were cultured on cover slips placed inside 6 multi-well culture plates, in serum-free medium for 24

h and then incubated with 1, 10 and 100 μM, and 1 mM CdCl2 for 48 h. After the exposure the cover slips were air dried and then fixed with Citofix (BD Biosciences, MA, USA). Successively the specimens were stained using the Papanicolaou method: briefly each specimen was hydrated in descending concentrations of 95% alcohol through 50% alcohol to distilled water for 2 min in each stage, then it was treated with Harris' Haematoxylin for 5 min, to stain the nuclei, rinsed in distilled water and differentiated in 0.5% aqueous hydrochloric acid (0,5% in 70% alcohol) for 1 minute to remove the excess stain. The specimen then was immediately rinsed in distilled water to stop the action of discoloration. For cytoplasmic staining each specimen was then dehydrated in ascending alcoholic concentrations from 70% through two changes of 80% alcohol for 2 min for each change and then treated with Papanicolaou Orange G6 for 5 min, rinsed in 90% alcohol (three changes for 1 minute for each change) and treated with eosin Azure 50 for 5 min. Each specimen was dehydrated in alcoholic concentrations from three changes of to 99% alcohol for 1 minute for each change. Finally the specimens were cleared in Xylene and mounted on microscope glass slides with Canada balsam. All reagents used were from Sigma Diagnostics, St. Louis, MO, USA. Specimens were then examined with an optical microscope (Nikon Instruments S.p.A., Milan, Italy) at different magnifications and images were acquired.

2.1.3 Cell proliferation

Cell proliferation was evaluated by Bromodeoxyuridine (BrdU) Cell Proliferation Assay (Calbiochem, San Diego, CA, USA).

Cells were seeded in complete medium at a density of $1x10^5$ in 96 multi-well culture dishes; then, after starvation, the medium was replaced by phenol red and serum-free medium containing different concentrations of CdCl2. Treatments with and without CdCl2 were carried out for 48 h. After 30 h from the CdCl2 exposure the BrdU solution was added to each specimen and the following procedure steps were performed according to the manufacturer's instruction. Absorbance in each well was measured using a Victor3 Multilabel Reader (Perkin Elmer, Boston, MA, USA) at wavelength of 450 nm.

2.1.4 SDS-PAGE, western blotting analysis, and immunoblotting

Cells were seeded in complete medium in 6 multi-well culture dishes; then, after starvation, the medium was replaced by phenol red and serum-free medium containing different concentrations of $CdCl_2$. Treatments with and without $CdCl_2$ were carried out for 48 h.

For VEGF, full length and cleaved PARP analysis, cells were homogenized in an ice-cold lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, 0.25% sodium dodecyl sulfate) supplemented with a

protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 15 min at 4°C at 10,000 *g*. The supernatant was collected and protein concentration was measured using a Coomassie Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Aliquots containing 20 μg of proteins were diluted in a 4X reducing Laemmli's sample buffer (250 mM Tris-HCl, pH 6.8, 20% glycerol, 8% SDS, 20% 2-mercaptoethanol, 0,008% bromophenol blue) and loaded into 10% SDS-PAGE. Then, proteins were transferred on polyvinylidene difluoride membranes (Hybond-P; Amersham Bioscience, Piscataway, NJ, USA). Membranes were blocked for 1 h at room temperature in 5% BSA-TTBS buffer (0.1% Tween 20, 20 mM Tris-HCl, 150 mM NaCl, pH 7,5), washed in TTBS, and incubated at 4°C overnight with primary antibodies: anti-VEGF, 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PARP, 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies were diluted in TTBS and incubations were followed by peroxidase-conjugated secondary IgG treatment (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Finally, the reacted proteins were revealed by the enhanced chemiluminescence system (ECL plus, Amersham Bioscience, Piscataway, NJ, USA). Image acquisition and densitometric analysis were performed with Quantity One software on a ChemiDoc XRS instrument (Bio-Rad Laboratories Inc.).

2.1.5 Angiogenesis (chicken embryo chorioallantoic membrane -CAM-assay)

Fertilized White Leghorn chicken eggs were incubated under routine conditions and a square window was opened in the egg shell at day 3 of incubation. Gelatin sponges (1 mm^3**)** were placed on the top of the CAM at day 8. The sponges were then absorbed with 5 μl of compounds to be tested. Sponges containing PBS were used as negative controls; prostaglandin E_1 (PGE_1) was used as stimulator of angiogenesis. MCF-7 and MCF-10A cells, untreated and treated with different concentrations of $CdCl_2$ for 48 h, were used at a concentration 50.000 cells/sponge. In each experiment we used six eggs per experimental point (*i.e.* six eggs treated with PBS, six eggs treated with PGE_1, and so on). Each experiment was repeated three times. Thus, the reported results refer to a total of 18 eggs per experimental point. Eggs were examined by microscopy, and positive angiogenesis was considered if new microvessels (in particular, microvessels surrounding the sponge, defined as circumfocal microvessels) had developed. Captured images were digitized and analysed for number of angiogenic blood vessels (*i.e.* those surrounding the sponges), using a customized image analysis software program (Scion Corporation, USA). Small (< 1 mm dia.), large (> 1 mm dia.), and tortuous microvessels were observed at the magnification x5. Angiogenesis was assessed by scoring the circumfocal microvessel number (CFMN).

Observers (two for each experiment), were blinded for what concerned the experimental conditions.

2.2 Cadmium and primary human neuroblast cell line FNC-B4

The primary human neuroblast long-term cell line FNC-B4 has been established, cloned, and propagated *in vitro* from human fetal olfactory epithelium [Vannelli *et al.*, 1995]. FNC-B4 cells synthesize both neuronal proteins and olfactory markers and respond to odorant stimuli, suggesting their origin from the stem cell compartment that generates mature olfactory receptor neurons. Moreover, this system represents a model of neurogenesis that, simplifying the cellular heterogeneity of the developing nervous system, may help in investigating *in vitro* pathologic perturbations of the activation, self renewal, differentiation, and survival of primary neuronal precursors. Thus, FNC-B4 cells represent a human *in vitro* model that can be useful in providing additional information on the effects of cadmium in neurons.

2.2.1 Cell culture and treatments

The primary human neuroblast long-term cell line FNC-B4 was isolated, cloned and propagated *in vitro* from human fetal olfactory neuroepithelium,

as described in Vannelli *et al.*, 1995. These cells express neuronal stem/differentiation markers indicating that they originate from the neuroblastic precursor compartment, which gives rise to mature neurons throughout life. FNC-B4 cells express olfactory specific markers: Olfactory Marker Protein (OMP; it is a phylogenetically conserved protein; its function has remained largely elusive); olfactory-type G protein (G_{olf}; it activates the lyase adenylate cyclase which converts ATP in cyclic AMP); Olfactory Cyclic Nucleotide-gated Channel (OCNC; it is a channel activated by binding of cAMP and conducting a depolarizing receptor current that leads to electrical excitation of the neuron); Olf-1 (an olfactory neuron-specific trans-acting factor capable of interacting with some olfactory neuron-specific genes). FNC-B4 were cultured in Coon's modification of F12 medium, supplemented with 10% foetal bovine serum and antibiotics. Materials for cell culture were bought from Invitrogen Co., Carlsbad, CA, USA. The FNC-B4 cell line grows as a monolayer, is non-tumorigenic and has a normal human kariotype. Cryogenically preserved, early passages of FNC-B4 cells are used in the present study.

FNC-B4 were switched, 24 h before the treatments, from complete culture medium to Coon's modification of F12 serum-free medium; then, at the beginning of each experiment, the medium was replaced with a Coon's modification of F12 serum-free medium containing 10 and 100 μM

cadmium chloride ($CdCl_2$; Sigma-Aldrich, St. Louis, MO, USA). Treatments with and without $CdCl_2$ are carried out for 24 h.

2.2.2 Phase-contrast light microscopy

For contrast phase microscopic observation, FNC-B4 cells were cultured on Petri dishes in serum-free medium for 24 h and then sham exposed or incubated with 10 and 100 μM $CdCl_2$. Cells were then viewed with a Nikon Microphot-FX microscope (Nikon Instruments S.p.A., Milan, Italy) at the original magnfication of x40.

2.2.3 Immunohistochemistry and confocal laser scanning microscopy

FNC-B4 cells were cultured on slides in the appropriate medium and then they were fixed with 3.7% paraformaldehyde (pH 7.4) for 10 min and permeabilized for 10 min with PBS containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). After rinsing in PBS, the slides were incubated with 2% bovine serum albumin for 15 min. Immunostaining was performed using the following primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA): vimentin antibody (V9; dilution 1:100; incubation overnight at 4°C); β-Tubulin III antibody (TU-20; dilution 1:100; incubation overnight at 4°C) and GFAP antibody (H-50;

dilution 1:500; incubation overnight at 4°C). Then, incubations with appropriate secondary conjugated antibodies were performed: A-11001 Alexa Fluor 488 goat anti-mouse IgG conjugated antibody (Invitrogen Co., Carlsbad, CA, USA) (dilution 1:200; incubation for 60 min at room temperature) for the detection of vimentin; R6393 rhodamine red goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR, USA) (dilution 1:200; incubation for 60 min at room temperature) for the detection of β-Tubulin III; A-11001 Alexa Fluor 488 goat anti-rabbit IgG (H + L) (Molecular Probes, Eugene, OR, USA) (dilution 1:200; incubation for 60 min at room temperature) for the detection of GFAP. Cells were then viewed by a Bio-Rad MCR 1024 ES confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA) equipped with a 15-mW Kr-Ar laser for fluorescence measurements and with differential interference contrast optics for transmission images. To minimize spectral bleed-through between the fluorescent channels, the emission of the different fluorochromes is measured by detecting the channels sequentially instead of simultaneously. Fluorescence was collected using a Nikon PlanApo x40 and x60 lens objective (Nikon Instruments SpA., Milan, Italy) and images analyzed by ImageJ software.

The percentage of cells positive to β-Tubulin III and/or to GFAP was calculated by counting the number of stained cells over the total cells in at

least 15 separate fields per slides. Each experiment was performed in triplicate.

2.2.4 Cell proliferation and cell viability

Cell proliferation was evaluated as incorporation of [^{3}H]thymidine (Amersham Bioscences, Little Chalfont, UK) in duplicating DNA. Cells were seeded in complete medium at a density of $7x10^4$ in 6 multi-well culture plates; then, control and treated cells, after starvation, were labelled with [^{3}H]thymidine (10 μCi/ml) at 37°C for 24 h. Cells were then harvested and incorporated radioactivity was measured by a liquid scintillation counter (Packard Instrument, Groningen, The Netherlands).

Cell viability was evaluated by a trypan blue exclusion test. 5 μl of trypan blue (0.5% in PBS) was added to 40 μl of resuspended cells. Trypan blue excluding (live) cells were counted in a hemocytometer in 3 randomly selected fields and averaged.

2.2.5 SDS-PAGE, western blotting analysis, and immunoblotting

For Nestin, β-tubulin III, full length and cleaved PARP analysis, the specimens were treated following the same procedure of the section 2.1.4.

In this study the following primary antibodies were used: anti-β-Actin, 1:10000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Nestin, 1:1000 (Chemicon, Temecula, CA, USA), anti-β-Tubulin III, 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-PARP, 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2.6 Quantitative real-time RT-PCR

RNA extraction. Total RNA was isolated from samples by "RNeasy Micro kit" (Qiagen S.p.A., Milan, Italy), according to the manufacturer's instructions. RNA concentration and purity were checked spectrophotometrically.

cDNA synthesis. RNA was reverse-transcribed using a commercial kit based on random primers technique ("Taqman Reverse Transcription Reagents", Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. The reaction was carried out in a final volume of 20 µl. The reaction mix contained: buffer 1X, $MgCl_2$ 5.5 mM, dNTP 2 mM, random hexamers 2.5 µM, RNAse inhibitor 0.4 U/µl, Reverse Transcriptase 1.25 U/µl. The reaction was performed with the following conditions: 25°C for 10 min, 48°C for 30 min and 95°C for 2 min.

Real time PCR. mRNA expression was measured by real-time PCR methods based on the use of Taqman probe. Reagents were purchased by

Applied Biosystems, Foster City, CA, USA, as pre-made kits: Nanog (Hs02387400_g1), CD15 (Hs00275643_s1), Nestin (Hs00707120_s1), CD271 (Hs00609976_m1), CD117 (Hs00174029_m1), GFAP (Hs00157674_m1), β-Tubulin III (Hs00801390_s1), D_2r (Hs00241436_m1), D_1r (Hs00265245_s1). In a total volume of 12.5 µl, the PCR mixture contained 6.25 µl of Universal Master Mix (Applied Biosystems), 0.625 µl of the ready-made specific probe and primers mix (Applied Biosystems) and 2.5 µl of cDNA. The thermal cycle conditions were as follows: 1 hold at 50 °C for 2 min, 1 hold at 95°C for 10 min and 45 cycles of a two step amplification protocol (95°C for 15 sec and 60°C for 1 min). All samples were analyzed in triplicate. Analysis of relative gene expression were obtained by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.2.7 Statistical analysis

The results were expressed as mean ± SEM. Comparison between groups was performed by the Mann-Whitney test or by the Wilcoxon test, as appropriate. $P<0.05$ was considered statistically significant.

2.3 Cadmium and human foetal spinal cord

Our current knowledge of the morphological and molecular mechanisms of CNS development stems largely from experiments in chick, rat, and mouse embryos as well as in *Xenopus* (Schoenwolf and Smith, 2000; Colas et al., 2001; Colas and Schoenwolf, 2003; Lawson et al., 2001; Ybot-Gonzalez et al., 2002; Copp et al., 2003; Gammill and Bronner-Fraser, 2002, 2003; Knecht and Bronner-Fraser, 2002; Basch et al., 2004; Farlie et al., 2004).

After Cd treatment, differences in life-stage specific teratogenic response have been observed in several mouse strains (Robinson et al., 2009); Cd exposure induced a greater reduction in expression of nervous system development-related genes indicating a role for Cd in neural tube defects and formation. In addition, Cd induces neurological deficits during early embryonic stages in zebrafish affecting the regionalization of the neural tube, the pattern formation and the cell fate determination, the commitment of proneural genes, and the induction of neurogenesis (Chow et al., 2008).

Several studies indicate that children of women exposed to Cd during pregnancy show lower motor and perceptual abilities, and that high Cd body burden in children is also related to impaired intelligence and lowered school achievement (Thatcher et al., 1982; Bonithon-Kopp et al., 1986). However, little is known about the molecular and cellular basis of developmental neurotoxicity of Cd in the sensitive early life stage of animals and humans. In order to investigate the effects of Cd in the human

CNS development, we focused on the role of Cd in affecting the morphology of the human foetal spinal cord during its morphogenesis.

2.3.1 Tissue collection

Twenty-five human foetal spinal cord samples (9-12 weeks gestational age) were collected after spontaneous or therapeutic abortions. Legal abortions were performed in authorized hospitals, and permission to collect tissue was obtained from the maternal donors at the end of the abortion procedure. The study protocols were approved by the University Ethical Committee (protocol no. 6783-04).

2.3.2 Organ culture

Each foetal spinal cord was isolated from spine and, immediately after collection, washed with phosphate-buffered saline (PBS) pH 7.4. The whole isolated spinal cord was observed by a stereomicroscope provided by a millimeteric scale. From the overlapping of the length of spine and spinal cord at this stage of development, the identification of lumbar tract was performed; the last 6 mm of the lumbar tract were collected and then cut in three fragments. Fragments were explanted onto a piece of Millipore

filter supported by a stainless grid platform in an organ-culture dish. One fragment was explanted into each dish. Organ cultures were grown in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA) serum free at 37°C in 5% CO_2 for 24 h. After removing the medium, the samples were incubated for 24 h in fresh medium in the presence or absence of increasing concentrations of $CdCl_2$ (10 µM, 100 µM). $CdCl_2$ solution (Sigma-Aldrich Corp.,St. Louis, MO, USA) pH 5.3 was diluted in the same serum-free culture medium used for the growth of the explants.

After incubation, some fragments were fixed in 4% buffered formalin, embedded in paraffin, and used for morphological detail and for immunohistochemical procedures. Other fragments were frozen and then processed for Western blot analysis.

2.3.3 Immunohistochemistry

Immunohistochemical studies were performed as described in the previous section 2.2.3. Gliosis represents a remarkable response of astrocytes to all types of injuries of the adult and developing CNS. One of the hallmarks of astrocyte activation and the resulting reactive gliosis is the upregulation of the intermediate filament system, mainly composed by glial fibrillary acidic protein (GFAP) (Pekny and Nillson, 2005). The immunohistochemical analysis of this marker was used to evaluate the

possible activation of gliosis induced by the exposure of the spinal cord samples to Cd. Furthermore, the immunohistochemical analysis of Choline Acetyltransferase (ChAT), a marker for cholinergic neurons in both peripheral and central nervous systems (Oda, 1999), was utilized to evaluate the effect of cadmium on the distribution and number of motor neurons in the spinal cord samples.

Briefly, transversal sections of human foetal spinal cord samples were incubated overnight at 4°C with the following primary antibodies: mouse monoclonal antibodies to human GFAP (clone 2A5, dilution 1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit policlonal antibody to human ChAT (dilution 1:150) (Abcam, Cambridge, UK). The sections were rinsed in PBS, incubated with biotinylated secondary antibodies followed by streptavidin-biotin peroxidase complex (Ultravision large volume detection system anti-polyvalent, Lab-Vision, Fremont, CA, USA). The reaction product was developed with the 3′,3′-diaminobenzidine tetrahydrochloride as chromogen (Sigma-Aldrich). The slides were examined with a Nikon Microphot-FX microscope (Nikon, Tokyo, Japan). The number of ChAT positive cells was counted in 15 separate fields for each slide of three independent experiments.

2.3.4 Western blot analysis

The foetal spinal cord samples were homogenized in an ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.25% sodium dodecyl sulfate) supplemented with a protease inhibitor cocktail (Sigma-Aldrich), and centrifuged for 15 min at 4°C at 10000g. The supernatant was collected and the protein concentration was measured using a Coomassie Bio-Rad protein assay kit (Bio-Rad Laboratories Inc.,Hercules, CA, USA). Aliquots containing 20 µg of proteins were diluted in 4X reducing Laemmli's sample buffer (250 mM Tris-HCl, pH 6.8, 20% glycerol, 8% SDS, 20% 2-mercaptoethanol, 0.008% bromophenol blue) and loaded onto 10% or 12% SDS-PAGE. Then, proteins were transferred on polyvinylidene difluoride membranes (Hibond-P, Amersham Bioscience, Piscataway, NJ). Membranes were blocked 1 h at room temperature in a 5% BSA-TTBS buffer (0.1% Tween-20, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5), washed in TTBS, and incubated at 4°C overnight with the following primary antibodies: anti-β actin (dilution 1:10000), anti-GFAP (dilution 1:1000), anti-β Tubulin III (dilution 1:2000), anti-Caspase 8 (dilution 1:600), anti-NGFRp75 (dilution 1:1000) and anti-PARP (dilution 1:1000) (Santa Cruz Biotechnology). GFAP and β Tubulin III were used for the quantification of the glial and neuronal components, respectively, in the control spinal cord samples and

after $CdCl_2$ exposure. The activation of the apoptotic pathway following $CdCl_2$ treatment was studied analysing NGFRp75, PARP and cleaved Caspase 8 expression (Pehar et al., 2007; Gulisano et al., 2009; Keane et al., 2001).

The incubation with the primary antibodies was followed by peroxidase conjugated secondary IgG treatment (Santa Cruz Biotechnology). Finally, the reacted proteins were revealed by the enhanced chemiluminescence system (ECL plus; Amersham Bioscience). Image acquisition and densitometric analysis were performed with Quantity One software on a ChemiDoc XRS instrument (Bio-Rad Laboratories Inc.). The densitometric analysis of each protein was normalized to β actin signal.

2.3.5 Apoptotic assays

The terminal deoxynucleotidyl transferase biotin-dUTP nick-end labelling (TUNEL) assay using the *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN) was utilized to detect apoptotic bodies at the single cell level of the human foetal spinal cords. Briefly, tissue sections were permeabilized with Proteinase K (20 μg/ml in 10mM Tris-HCl, pH 7.6) for 15 min and stained with the TUNEL mixture according to the manufacturer instructions. Nuclei were counterstained with DAPI (4-6'-diamidino-2-phenylindole; Invitrogen). Slides were visualized using a

fluorescence microscope (Nikon Microphot-FX microscope, Nikon). The number of TUNEL positive cells (green) colocalized with DAPI (blue), resulting in a cyan colour, was counted in 15 separate fields for each slide of three independent experiments.

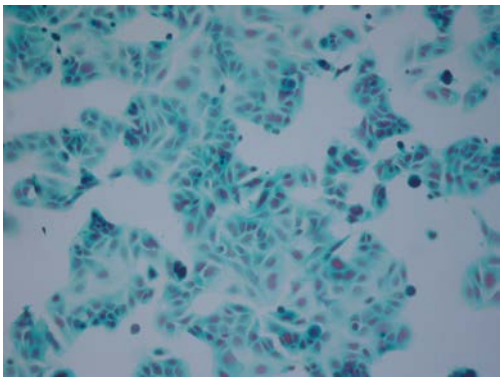
2.3.6 Statistical analysis

Results are expressed as mean $\pm$ SD. Comparison between groups was performed by the Mann-Whitney test or by the Wilcoxon test, as appropriate. $P<0.05$ or $P<0.01$ were considered statistically significant.

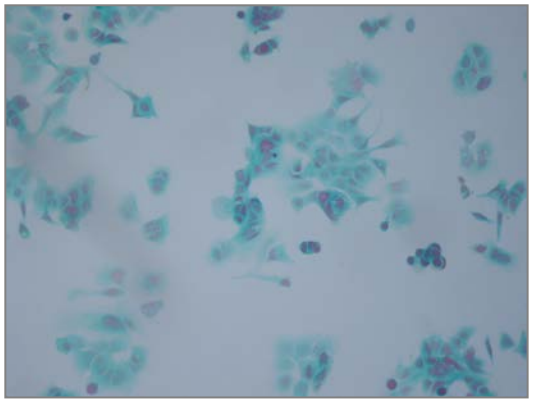
3 RESULTS

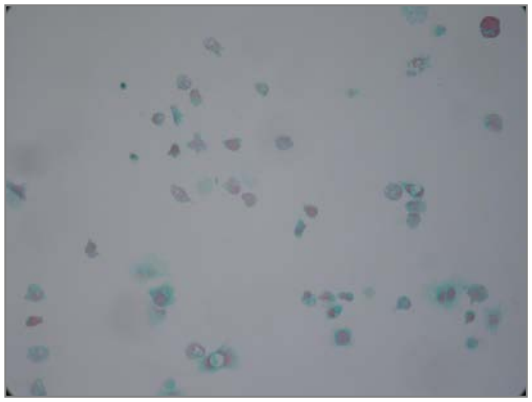
3.1 Cadmium and normal/tumour human breast cell lines (MCF-7 and MCF-10A respectively)

Cell morphology. Observation by light microscopy reveals that 1 μM $CdCl_2$-treated MCF-7 and MCF-10A cells (Fig.6 B, Fig.7 B) don't show evident alterations in size and shape in comparison to control, untreated cells (Fig.6 A, Fig.7 A). The 1 μM $CdCl_2$ exposure don't affect the characteristic growth of both cell lines. The 10 μM $CdCl_2$ exposure reveals a modifications in size, shape a growth of some of MCF-7 and MCF-10A cells in comparison to control (Fig.6 C, Fig.7 C). Some of the cells are distributed as small groups or separately. They appear smaller than the untreated cells and their shape changes to spherical morphology. These alterations are more evident in MCF-10A in comparison to MCF-7. The 100 μM $CdCl_2$ exposure reveals deep modifications in both the cell lines (Fig.6 D, Fig.7 D). Only few separate cells are observable; they appear smaller than untreated cells and with spherical morphology. The 1 mM $CdCl_2$ exposure reveals a large amount of cadmium precipitate that don't allow a right evaluation of this exposure (Fig.6 E, Fig.7 E); probably the cadmium precipitate can not allow it to fully act on the cells.

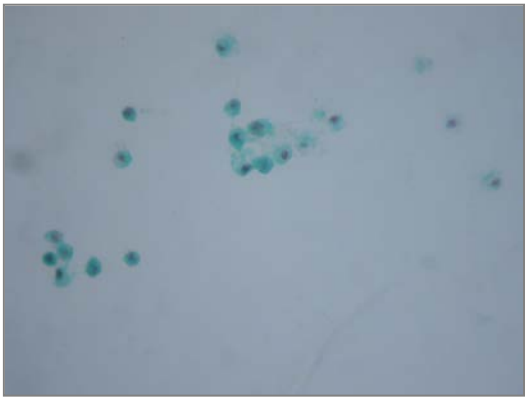


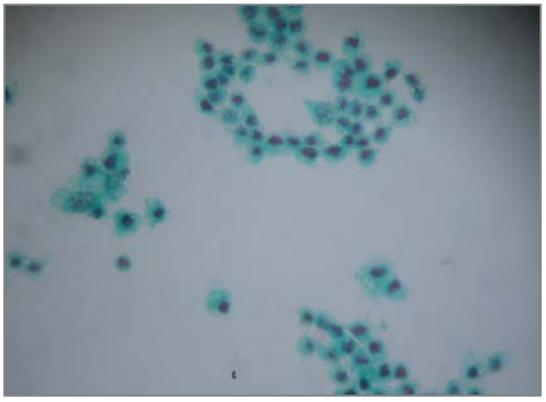
A





B **C**



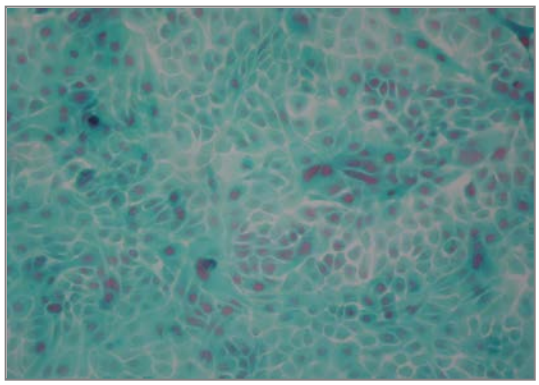


D **E**

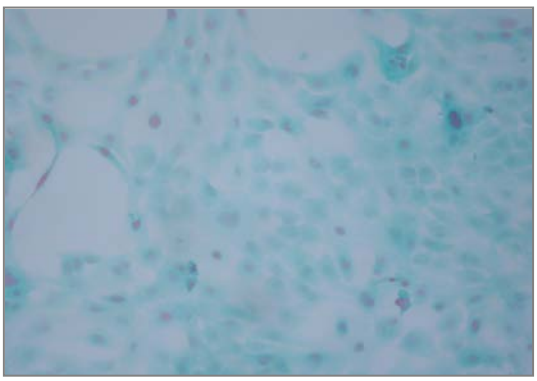
Figure 6. MCF-7 cell morphology before and after stimulation with $CdCl_2$.

A: control, untreated cells at different magnifications. Cells show the typical MCF-7 characteristics: growth in monolayer with dome structures, polyhedric shape, sometimes bi- or multinucleated. B: MCF-7 cells treated with $CdCl_2$ 1 μM. No alterations in size and morphology are evident if compared to control cells. C: MCF-7 cells treated with $CdCl_2$ 10 μM. Some of the cells are distributed separately or as small groups. Cells are smaller if compared to untreated ones and

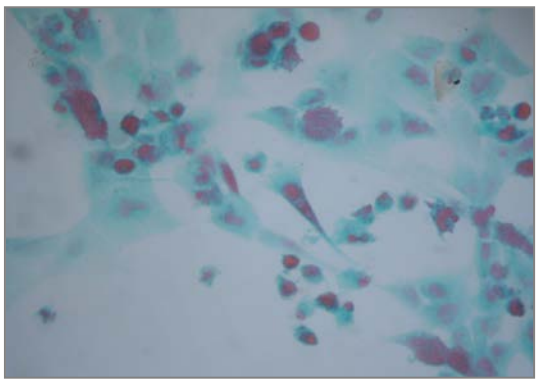
with spherical shape. D: MCF-7 cells treated with $CdCl_2$ 100 μM. Only few little separate cells with spherical shape are observable. E: MCF-7 cells treated with $CdCl_2$ 1 μM. A large amount of cadmium precipitate don't allow a right evaluation of this exposure. Total magnification x200.



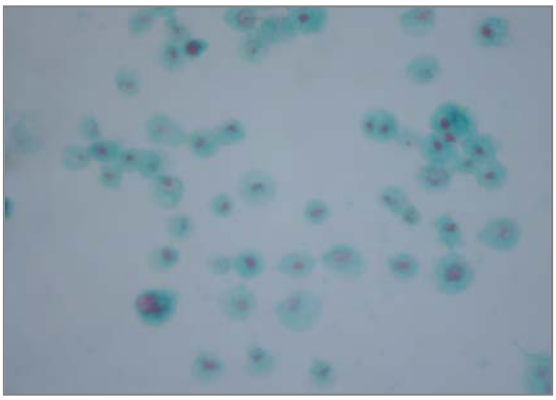
A



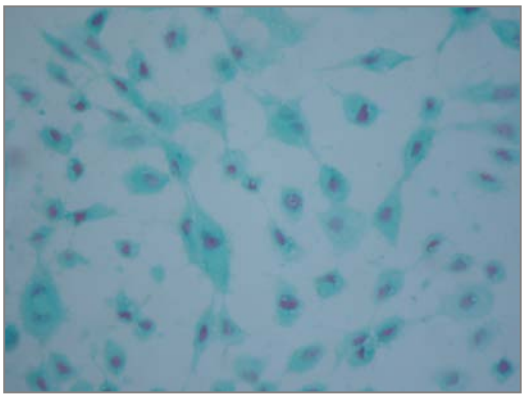
B



C



D



E

Figure 7. MCF-10A cell morphology before and after stimulation with $CdCl_2$.

A: control, untreated cells at different magnifications. Cells show the typical MCF-10A characteristics: growth in monolayer, polyhedric shape, single spherical nucleus. B: MCF-10A cells treated with $CdCl_2$ 1 μM. No alterations in size and morphology are evident if compared to control cells. C: MCF-10A cells treated

with $CdCl_2$ 10 μM. The cells are distributed as small groups. Some of the cells are smaller in comparison with untreated ones and with spherical shape. In some of the cells are present thin cytoplasmic extroflessions and nuclei with irregular shape. D: MCF-10A cells treated with $CdCl_2$ 100 μM. Only few separate little cells with spherical shape are observable. E: MCF-10A cells treated with $CdCl_2$ 1 μM. A large amount of cadmium precipitate don't allow a right evaluation of this exposure. A and B, total magnification x200; C, D and E, total magnification x400.

Cell proliferation.

The results of the BrdU proliferation assay show an evident inhibitory effect of $CdCl_2$ exposure on both MCF-7 and MCF-10A cell proliferation. In both the cell lines, the 1 μM $CdCl_2$ exposure don't affect significantly the cell proliferation in comparison to control, untreated cells. The 10, 100 μM and 1 mM $CdCl_2$ exposures inhibits significantly both MCF-7 and MCF-10A cell proliferation in a dose-dependent manner (Figg. 8 and 9).

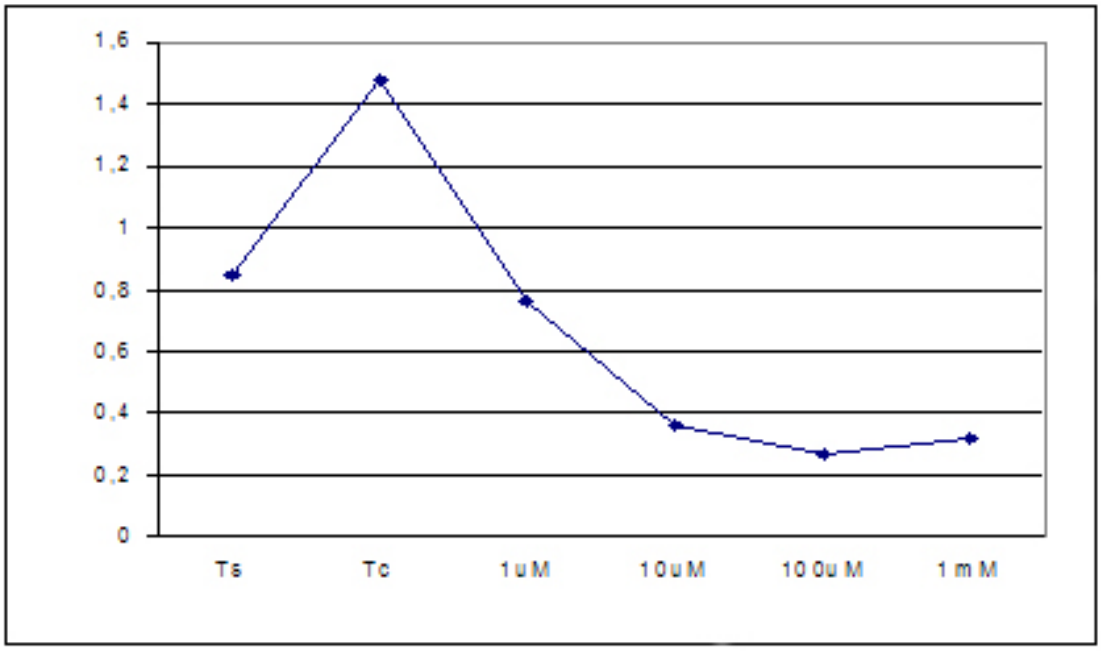


Figure 8. Proliferation graph of MCF-7 cells exposed at different $CdCl_2$ concentrations. In x-axis, $CdCl_2$ concentrations; Ts = control, untreated cells cultured in serum-free medium without phenol red, Tc = cells cultured in complete medium with serum. In y-axis, optical density (OD) values at 450 nm. $CdCl_2$ 1 μM exposure don't affect significantly the proliferation of MCF-7 cells in comparison to control, untreated cells. $CdCl_2$ 10, 100 μM and 1 mM exposures deeply decrease the cell proliferation.

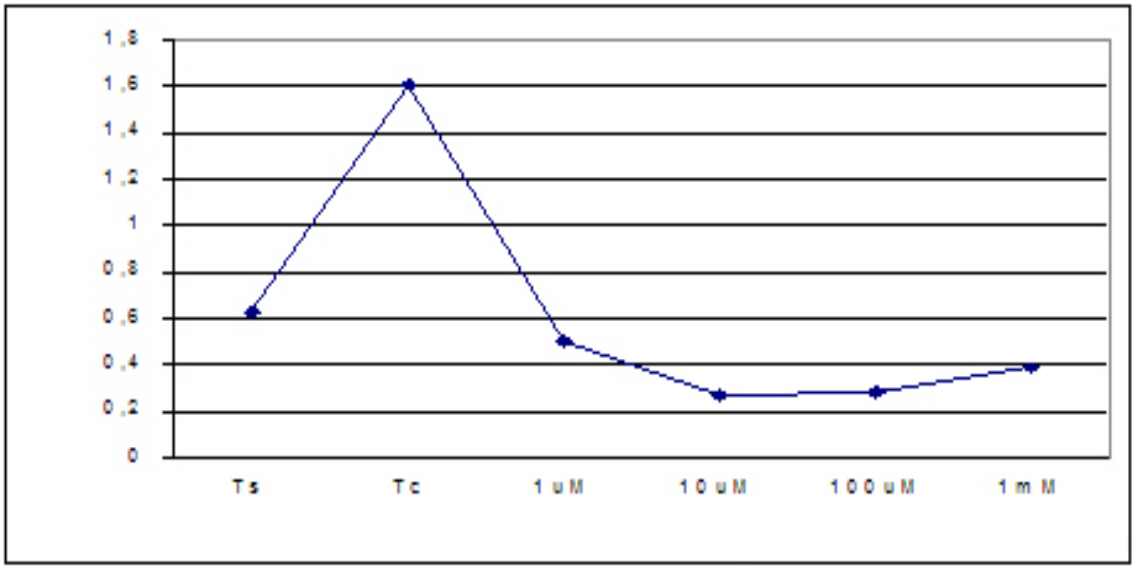


Figure 9. Proliferation graph of MCF-10A cells exposed at different $CdCl_2$ concentrations. In x-axis, $CdCl_2$ concentrations; Ts = control, untreated cells cultured in serum-free medium without phenol red, Tc = cells cultured in complete medium with serum. In y-axis, optical density (OD) values at 450 nm. $CdCl_2$ 1 μM exposure don't affect significantly the proliferation of MCF-10A cells in comparison to control, untreated cells. $CdCl_2$ 10, 100 μM and 1 mM exposures deeply decrease the cell proliferation.

PARP-mediated apoptotic pathway. In order to determine if the treatment with different concentrations of $CdCl_2$ can affect the expression of protein involved in DNA repair and in programmed cell death, we investigated the expression of poly (ADP-ribose) polymerase (PARP), a marker of caspase 3-mediated pathway of apoptosis. Human PARP is a 116 kDa nuclear protein involved in repair of DNA nicks induced by various stressors and it is one of the substrates for caspase 3, which cleaves PARP at Asp 214 and Gly 215 leading to formation of 85 kDa and 25 kDa fragments during

apoptosis. Cleavage of PARP correlates with DNA fragmentation and other morphological changes making it a critical marker of apoptosis.

The results obtained show that 1 μM $CdCl_2$ exposure don't affect the PARP-mediated levels of apoptosis in MCF-7 cells but increases significantly the apoptosis in MCF-10A cells in comparison to control untreated cells. 10 and 100 μM $CdCl_2$ exposure increases significantly the PARP-mediated apoptosis in MCF-10A cells in a dose-dependent manner. The 10 μM $CdCl_2$ exposure increases significantly also the PARP-mediated apoptosis of MCF-7 cells whereas the 100 μM $CdCl_2$ exposure shows a decreases of levels of apoptosis probably because of the presence of a cadmium precipitate that don't allow the metals to fully affect the cells (Figg. 10 and 11).

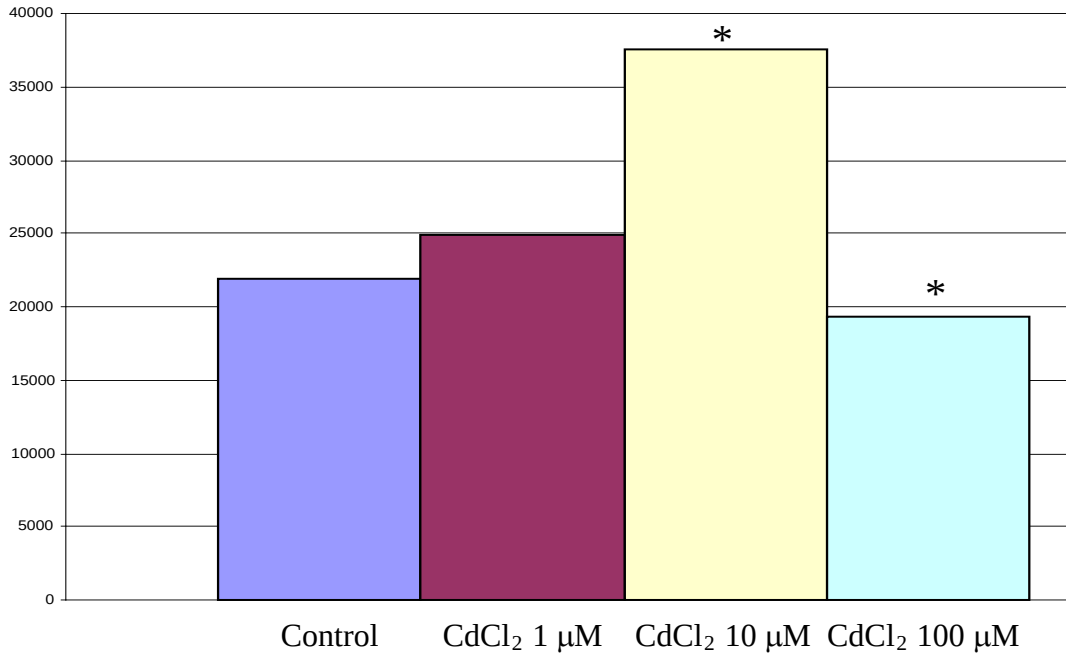


Figure 10. Graph of densitometric analysis of anti-PARP western blot in MCF-7 cells exposed at different $CdCl_2$ concentrations. In y-axis number of pixel measured by appropriate image elaboration software. The 10 and 100 μM $CdCl_2$ exposure increases significantly the PARP-mediated apoptosis of MCF-7 cells in a dose-dependent manner.

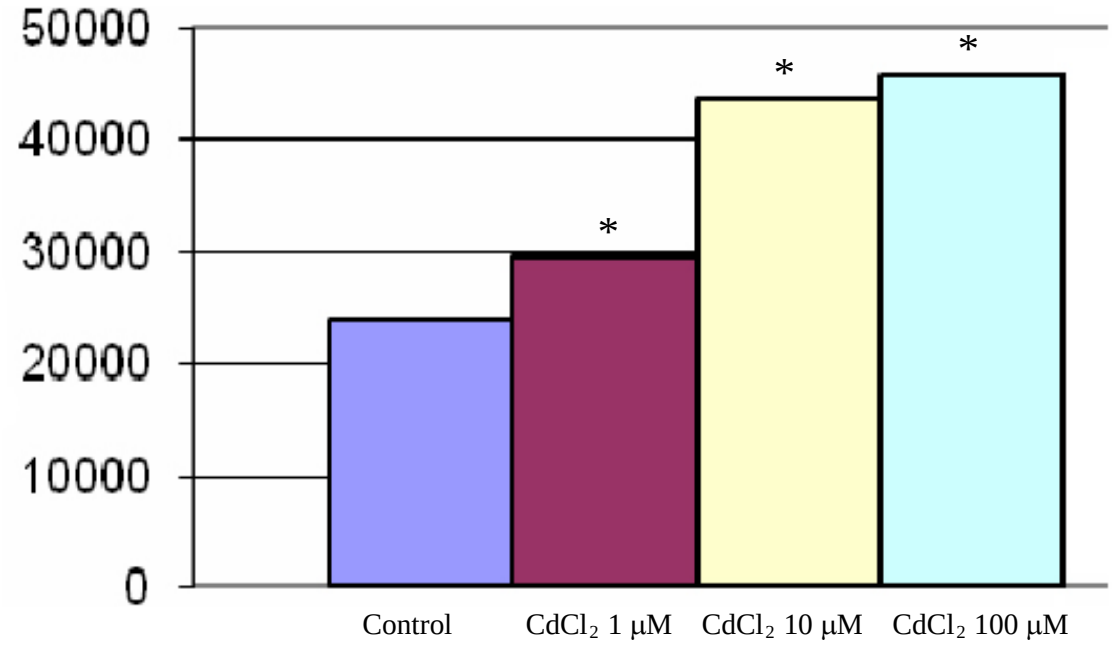


Figure 11. Graph of densitometric analysis of anti-PARP western blot in MCF-10A cells exposed at different $CdCl_2$ concentrations. In y-axis number of pixel measured by appropriate image elaboration software. The 1, 10 and 100 µM $CdCl_2$ exposure increases significantly the PARP-mediated apoptosis of MCF-10A cells in a dose-dependent manner.

Angiogenic potential and Vascular Endothelial Growth Factor (VEGF) levels. The chicken embryo chorioallantoic membrane assay has been a model for studying neovascularization since the early 1970's. It is perhaps the most widely used in vivo assay to assess the anti-angiogenic or angiogenic activity of test substances. The early chick embryo lacks a mature immune system and was therefore used to study tumor-induced angiogenesis. The basic assay is performed by implanting a membrane or coverslip or sponge containing the compound of interest on the chick

embryo chorioallantoic membrane through a hole cut in the egg shell. This causes a typical radial rearrangement of vessels towards, and a clear increase of vessels around the sponge within four days after implantation. Blood vessels entering the sponge are counted under a stereomicroscope. The CAM assay is relatively simple and inexpensive and thus suitable for large-scale screening. The major disadvantage of this assay is that the CAM contains already a well-developed vascular network, which makes it difficult to discriminate between new capillaries and already existing ones.

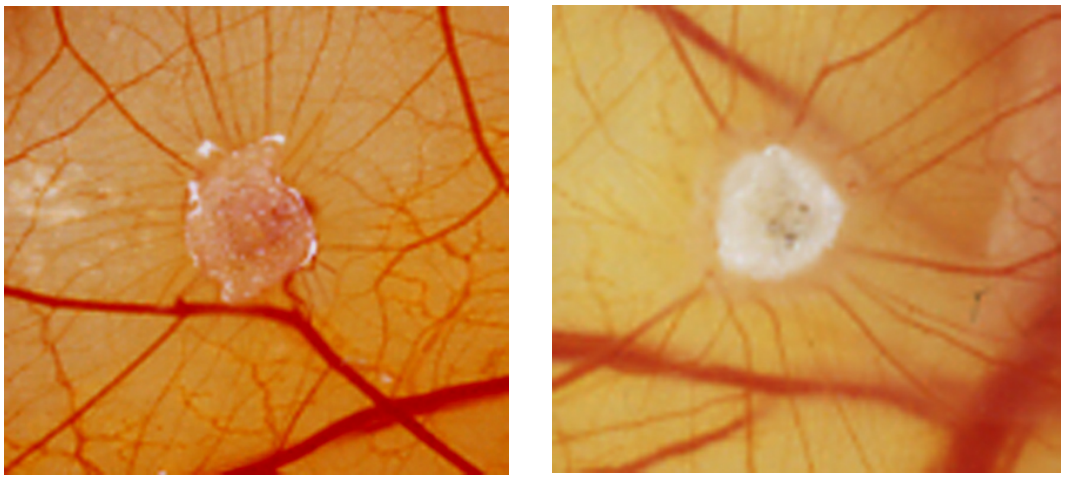


Figure 12. Examples of sponge containing PGE_1 implanted in CAM surrounded by numerous circumfocal microvessels.

The CAM assay was performed testing untreated MCF-7 cells and, untreated, 1 and 10 μM $CdCl_2$-treated MCF-10A cells. The untreated MCF-7 cells show a strong angiogenic response if compared to very strong one induced by PGE_1; it was due to proper characteristics of this cancer

cell line. In MCF-10A cells, 1 μM $CdCl_2$ exposure don't affect their angiogenic potential in comparison to that induced by untreated cells, whereas 10 μM $CdCl_2$ exposure induces an angiogenic response similar to that induced by untreated MCF-7 cells (Table 3).

MCF 7	
Applied stimulus	*Circumfocal microvessels average*
Cells cultured for 48 h in complete medium with serum	**26.3**
PGE_1	>30

MCF-10A Cells	
Applied stimulus	*Circumfocal microvessels average*
Cells cultured for 48 h in complete medium with serum	14.8
Cells cultured for 48 h in serum-free medium without phenol red	10
1 μM CdCl2-treated cells	12.3
10 μM CdCl2-treated cells	**24.8**

Table 3. CAM assay. Summary of circumfocal microvessels count after implantation of sponge containing untreated MCF-7 and, untreated, 1 and 10 μM CdCl2-treated MCF-10A cells.

In order to evaluate if the angiogenic response induced in MCF-10A cells by 10 μM $CdCl_2$-exposure was associated to an increased level of VEGF, western blot analysis was performed before and after exposure of MCF-10A cells for 48 h at different concentrations of $CdCl_2$.

The results show that 1, 10 and 100 $CdCl_2$-exposure induces a significant increasing of VEGF levels in a dose-dependent manner, confirming the hypothesis that the increasing angiogenic potential of MCF-10A cells treated with $CdCl_2$ is associated with a parallel increasing of VEGF levels (Fig. 13).

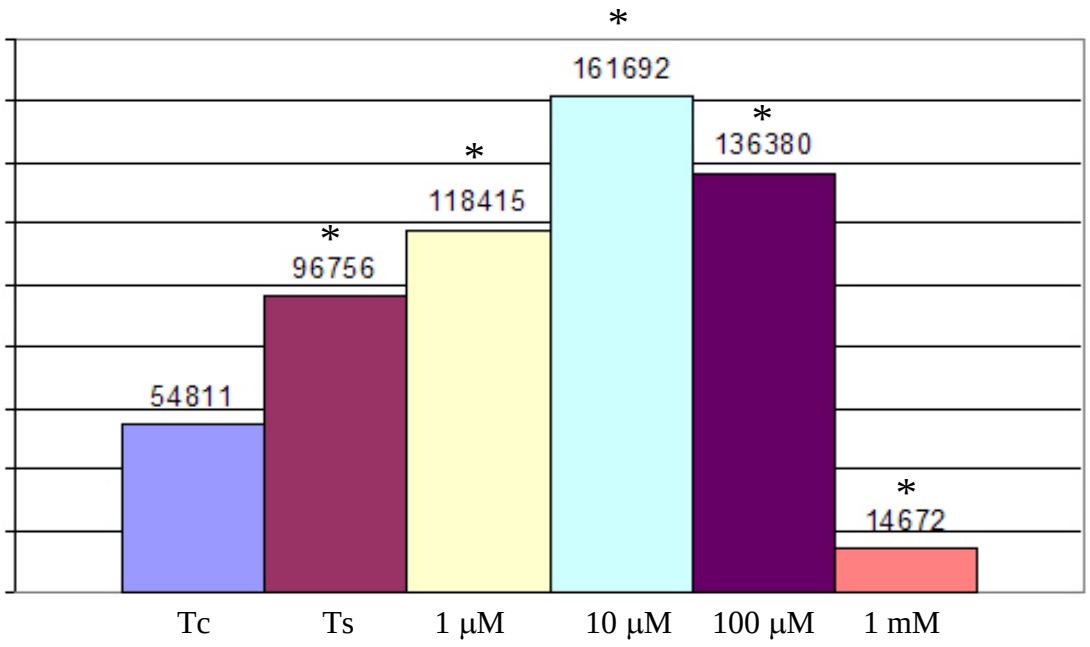
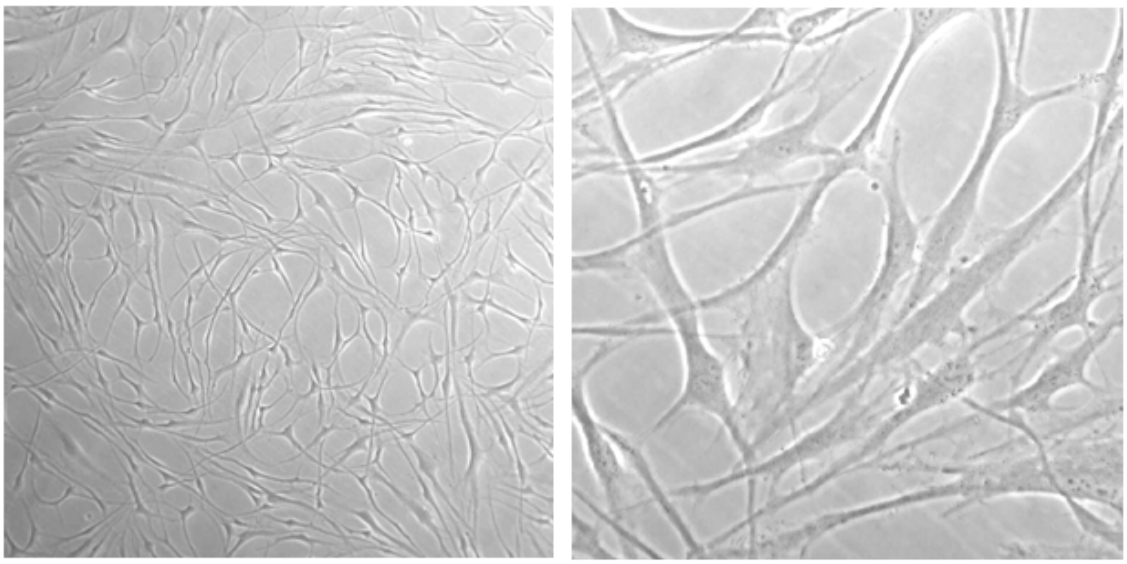


Figure 13. Graph of densitometric analysis of anti-VEGF western blot in MCF-10A cells exposed at different CdCl2 concentrations. In x-axis, $CdCl_2$ concentrations; Ts = control, untreated cells cultured in serum-free medium without phenol red, Tc = cells cultured in complete medium with serum. In y-axis number of pixel measured by appropriate image elaboration software. The 1, 10

and 100 μM $CdCl_2$ exposure increases significantly the VEGF levels in MCF-10A cells in a dose-dependent manner. The 1 mM $CdCl_2$ exposure results are not rightly valuable because of the cadmium precipitate affecting the specimen.

3.2 Cadmium and primary human neuroblast cell line FNC-B4

FNC-B4 cell morphology. Observation by contrast phase light microscopy reveals that 10 μM $CdCl_2$-treated cells (Fig.6, panels C and D) show a bulky body shape and long neuritic processes analogue to control, untreated cells (Fig. 6, panels A and B); synaptic bundles are well represented and manifold intercellular contacts are clearly evident. After 100 μM $CdCl_2$ exposure, cell morphology appears significantly modified (Fig. 6, panels E and F); cells are stretched and thinner even though neurites are still present, normal in length, and there are numerous synaptic bundles.



A B

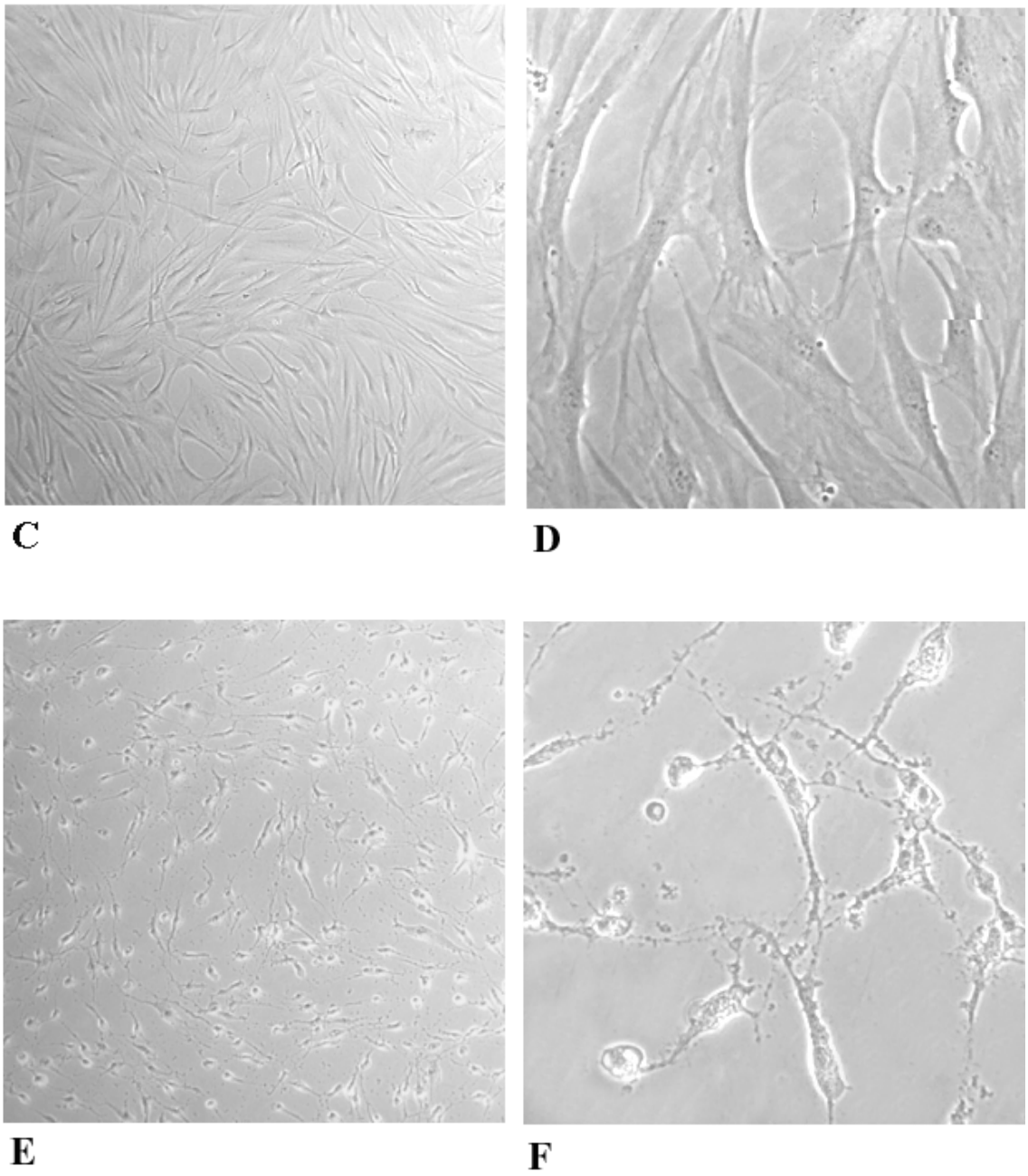


Figure 6. FNC-B4 cell morphology before and after stimulation with $CdCl_2$.
Panels A and B: control, untreated cells at different magnifications. Cells show the typical neuronal morphology with neuritic processes and manifold intercellular contacts. Panels C and D: 10 µM $CdCl_2$-treated neuroblasts. Cell shape and synaptic network appear unaffected by the treatment in comparison to control neuroblasts. Panels E and F: 100 µM $CdCl_2$-treated neuroblasts. The exposure induces changes of the polyedric cell shape so that FNC-B4 neurons acquired a spindle shape morphology; cytoplasmic processes are well represented.
Contrast phase light microscopy. A, C and E panels: total magnification x40. B, D and F panels: total magnification x400.

Cytoskeleton and vimentin network organization. Analysis by confocal laser scanning microscopy shows that exposure of FNC-B4 human neuroblasts to a high concentration of $CdCl_2$ results in significant cytoskeletal network modifications. In fact, after 10 μM $CdCl_2$ exposure, vimentin appears unaffected in its distribution and organization (Figure 7, panels B) in comparison to untreated cells (Fig. 7, panels A); both in 10 μM $CdCl_2$-treated and in control cells, vimentin is evident and uniformly distributed in the cell bodies and in the neurites. Instead, after 100 μM $CdCl_2$ exposure (Fig. 7, panels C), vimentin appears accumulated and aggregated in neuronal cell bodies and axons giving FNC-B4 cells a deeply modified cytoskeletal phenotype. Both control and treated cells (with low and high concentrations of $CdCl_2$) show branched neurites and several synaptic contacts, considered hallmarks of neuronal plasticity and of differentiation (Cowen and Gavazzi, 1998).

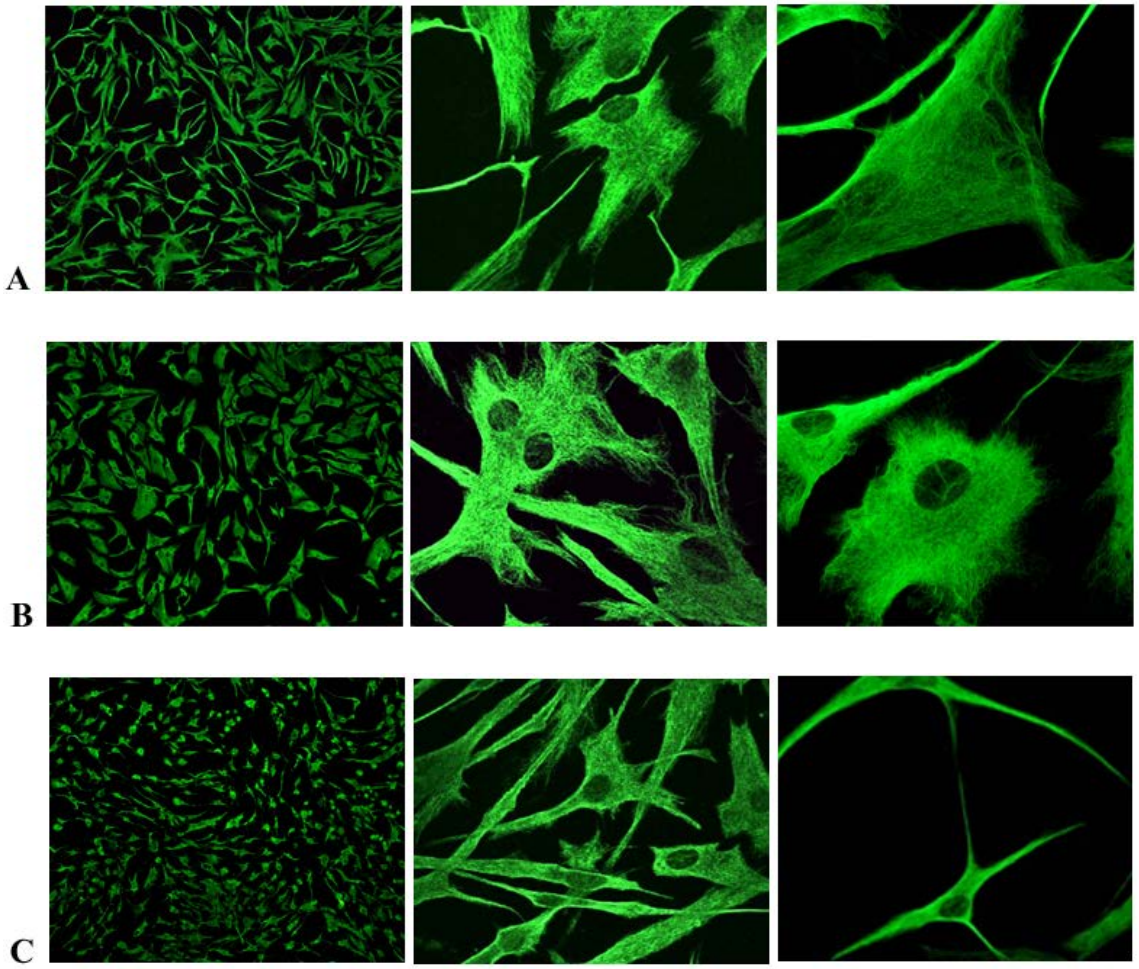


Figure 7. FNC-B4 cytoskeletal modifications before and after stimulation with $CdCl_2$. Panels A: control, untreated cells at different magnifications. Vimentin, revealed by appropriate primary and fluorescent secondary antibodies, appears in green. Fluorescence appears uniformly distributed in the cell bodies and outgrowths. Panels B: 10 µM $CdCl_2$-treated cells. $CdCl_2$ treatment does not significantly affect vimentin distribution and architecture.

Panels C: 100 µM $CdCl_2$-treated cells. Vimentin appears denser and closely packed in the cell body and in the outgrowths in comparison to control and to 10 µM $CdCl_2$-treated cells. Immunohistochemistry and confocal laser scanning microscopy. Total magnification in each panel: from left to right x40, x400 and x600, respectively.

Cell proliferation and viability. In order to investigate the role of $CdCl_2$ on cell proliferation, we measured the [^{3}H]thymidine incorporation in control and treated cells. Neuroblasts, treated with 10 μM $CdCl_2$, have a statistically significant increase of [^{3}H]thymidine incorporation in comparison to control, untreated cells (Table 3). In contrast, 100 μM $CdCl_2$-treated FNC-B4 cells, show a significant decrease in the incorporated radioactivity, in comparison both to control and to 10 μM $CdCl_2$-treated cells (Table 3). Cell viability, measured by trypan blue exclusion assay, is not significantly affected by 10 μM $CdCl_2$ treatment in comparison to control, untreated cells. When cells were exposed to 100 μM $CdCl_2$, the number of trypan blue positive cells increased (15%, $p<0.05$).

Treatments	Cell Proliferation
10 μM $CdCl_2$	119 ± 6
100 μM $CdCl_2$	23 ± 7

Table 3. Cell proliferation is assessed by [^{3}H]thymidine incorporation in FNC-B4 cells stimulated with increasing concentrations of $CdCl_2$. Results are expressed as percent increase over the control value (100%); each value of n=3 experiments represents the mean ± SEM.

PARP-mediated apoptotic pathway. In order to determine if the treatment with different concentrations of $CdCl_2$ can affect the expression of protein involved in DNA repair and in programmed cell death, we investigated the expression of poly (ADP-ribose) polymerase (PARP), a marker of caspase 3-mediated pathway of apoptosis. Human PARP is a 116 kDa nuclear protein involved in repair of DNA nicks induced by various stressors and it is one of the substrates for caspase 3, which cleaves PARP at Asp 214 and Gly 215 leading to formation of 85 kDa and 25 kDa fragments during apoptosis. Cleavage of PARP correlates with DNA fragmentation and other morphological changes making it a critical marker of apoptosis. In FNC-B4 cells treated with $CdCl_2$ the PARP-mediated apoptotic pathway is activated in a dose-dependent manner: the cleaved form of PARP progressively increases (and the full length of PARP decreases) when cells are treated with 10 and 100 μM $CdCl_2$ in comparison to control, untreated cells (Fig. 8).

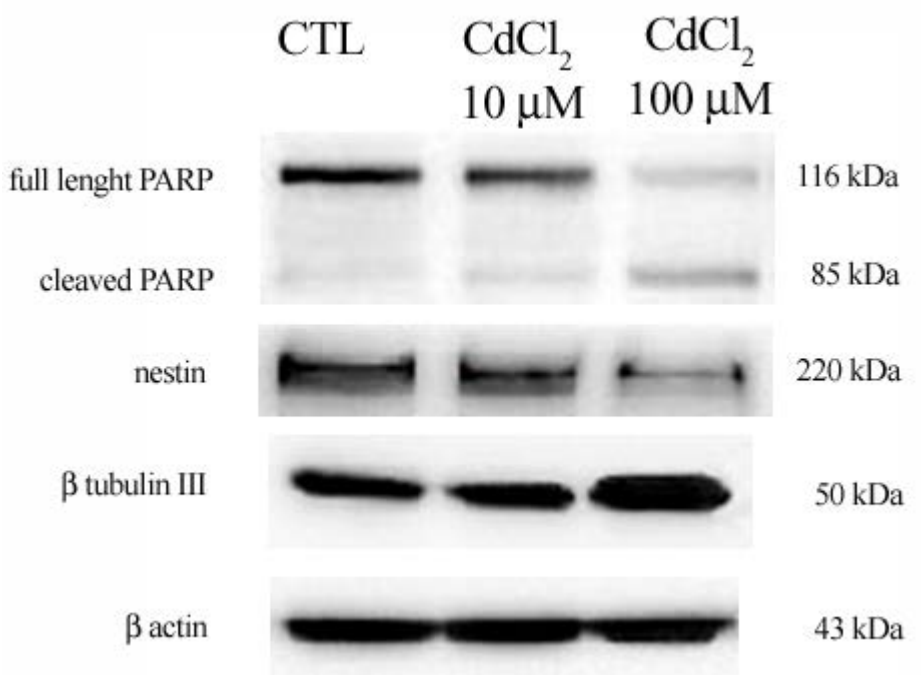


Figure 8. Western blotting analysis of Nestin, β-Tubulin III, and PARP in FNC-B4 neuroblsts before and after stimulation with $CdCl_2$. Western blotting analysis for Nestin and β-Tubulin III expression reveals bands migrating approximately at the expected 220 and 50 kDa molecular sizes, respectively. With the increasing of $CdCl_2$ doses, the expression of Nestin progressively decreases whereas the expression of β-Tubulin III increases. Western blotting analysis for full length (116 kDa) and cleaved (85 kDa) PARP shows a significant increase in the cleaved form expression when FNC-B4 cells are treated with 100 µM $CdCl_2$.

Gene and protein expression. FNC-B4 cells, obtained from the human fetal olfactory epithelium, have at the same time the properties of immature neurons and the ability to differentiate and express both neuronal proteins as well as olfactory specific markers (Vannelli et al., 1995). When FNC-B4 cells are exposed to 10 and 100 µM $CdCl_2$, the expression of several somatic markers such as Nanog (a homeodomain transcription factor, marker of pluripotency), CD15 (a carbohydrate antigen associated to the

extracellular matrix and marker of highly proliferative cells) and Nestin (a protein associated with neural stem cell) appear significantly decreased, with a concentration-dependent pathway, in comparison to untreated, control cells (Fig. 9, A). Nevertheless, after 10 and 100 μM $CdCl_2$ treatments, GFAP (glial fibrillary acid protein) (Fig. 9, B) and β-Tubulin III (Fig. 9, C) expression increase, suggesting the possibility of a multi-differentiation into different cell subtypes, neurons or astrocytes. In order to confirm the trend of FNC-B4 cells that differentiate in response to $CdCl_2$ exposure, the expression of β-Tubulin III and Nestin proteins was evaluated. Western blotting analysis confirmed that while β-Tubulin III expression increases in a dose-dependent manner, the expression of Nestin, significantly decreases (Fig. 8).

Since the simultaneous over expression of GFAP and β-Tubulin III was somewhat intriguing, we analyzed, by confocal laser scanning microscope, the localization of GFAP and β-Tubulin III in control and treated cells. Data obtained demonstrated that GFAP and β-Tubulin III co-localize in $CdCl_2$ treated cells, with a dose-dependent pathway (Fig. 10). In particular, control cells (Fig. 10, A, B, C and J) are positive to β-Tubulin III (43.38 ± 2.83 %, n=3) and virtually to GFAP (0.42 ± 0.12 %, n=3); 10 μM $CdCl_2$ treated cells (Fig. 10, D, E, F and J) are positive both to β-Tubulin III (66.42 ± 3.24 %, n=3) and to GFAP (7 ± 2 %, n=3). Treatment with 100 μM $CdCl_2$ (Fig. 10, G, H, I and J) increased the number of cells positive to β-Tubulin III (93.7 ± 3.8 %, n=3) and GFAP (47 ± 3 %, n=3). After 10 and 100 μM $CdCl_2$ treatment the co-localization of β-Tubulin III and GFAP in FNC-B4 neuroblasts was 7 ± 2 % (n=3) and 47 ± 3 % (n=3) respectively. In the merged images (Fig. 10, C, F, I and J) the yellow colour indicates a co-localization of β-Tubulin III with GFAP in the treated cells.

Among cell markers suggesting a proliferative cell phenotype, we analyzed the expression of CD117 gene (encoding a receptor for stem cell factor)

since it identifies and characterizes neurons with proliferative and stem phenotypes. CD117 is over-expressed in FNC-B4 cells treated with 10 μM $CdCl_2$ while the decrease of expression of this marker is dramatic when cells are stimulated with 100 μM $CdCl_2$ (Fig. 11, A). Since dopamine, in parallel to its action in odour processing, plays a growth factor-like role in the permanent neurogenesis observed in the olfactory epithelium, by a direct dopamine D_1receptor (D_1r) or D_2receptor (D_2r)-mediated action, we evaluated the expression levels of dopamine D_1r and dopamine D_2r before and after $CdCl_2$ treatments. Our data showed that $CdCl_2$ induced on FNC-B4 neuroblasts an increase of dopamine D_1r expression in a dose-dependent pathway (Figure 11, B); on the contrary, dopamine D_2r expression dramatically decreased after stimulation both with 10 and 100 μM $CdCl_2$ (Fig. 11, C).

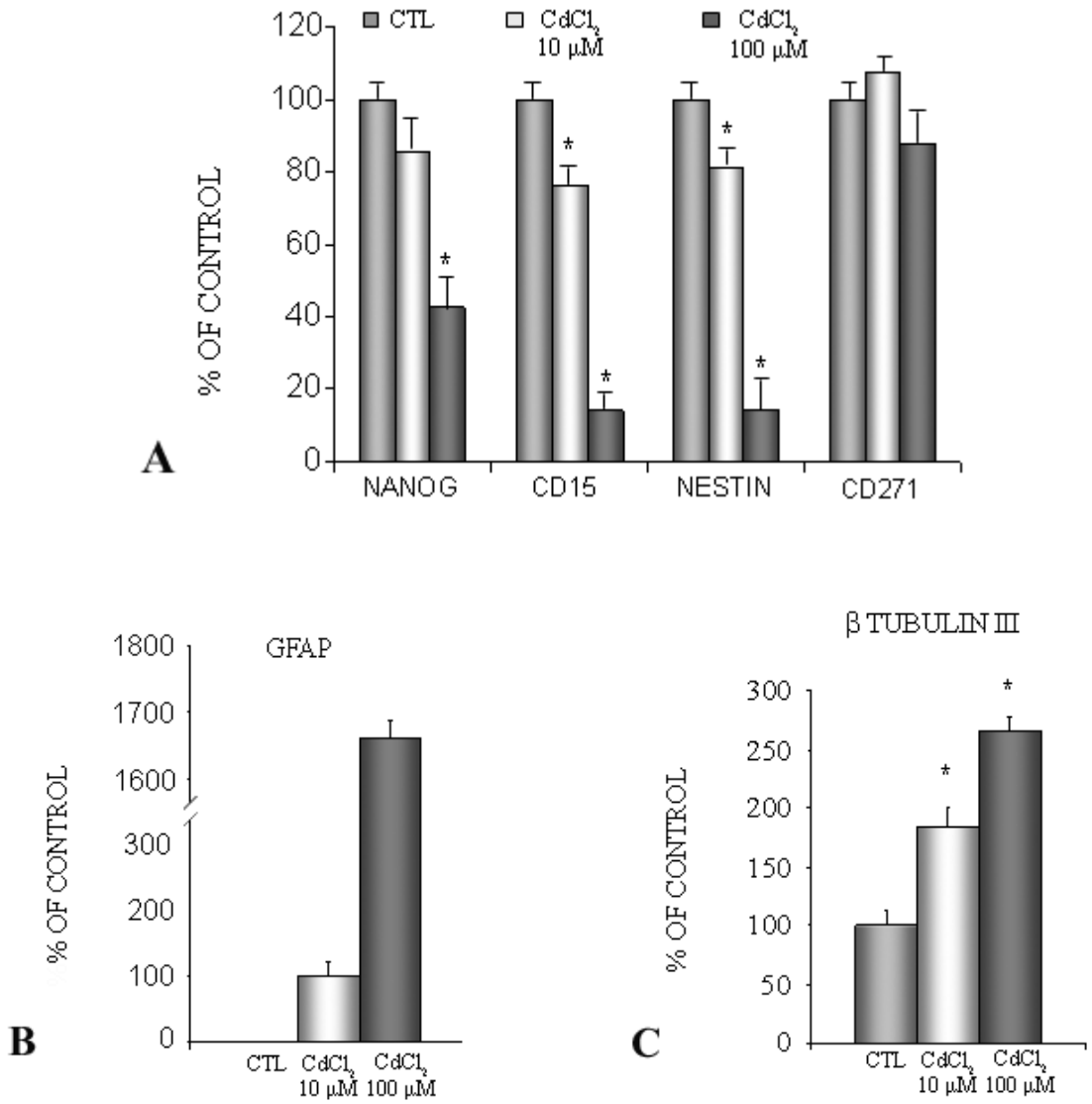


Figure 9. Quantitative gene expression analysis (Nanog, CD15, Nestin, GFAP and β-Tubulin III) in FNC-B4 before and after stimulation with $CdCl_2$. A: mRNA expression of neural markers. Nanog, CD15 and Nestin decrease in 10 and 100 µM $CdCl_2$-treated neuroblasts. mRNA expression of CD271 (nerve growth factor receptor), is high both in control (CTL) and in treated cells, confirming the viability of control and CdCl2-exposed neuroblasts. B: mRNA expression of GFAP progressively increases when FNC-B4 cells are stimulated with 10 and 100 µM $CdCl_2$. C: mRNA expression of β-Tubulin III increases when neuroblasts are stimulated with 10 and 100 µM $CdCl_2$. Results are calculated accordingly to the comparative cycle threshold method using the GAPDH as reference gene for normalization and are expressed as percentage of data obtained in the untreated, control cells (CTL). Data are reported as mean ± SEM from at least three separate experiments; * $p<0.05$ *vs.* control.

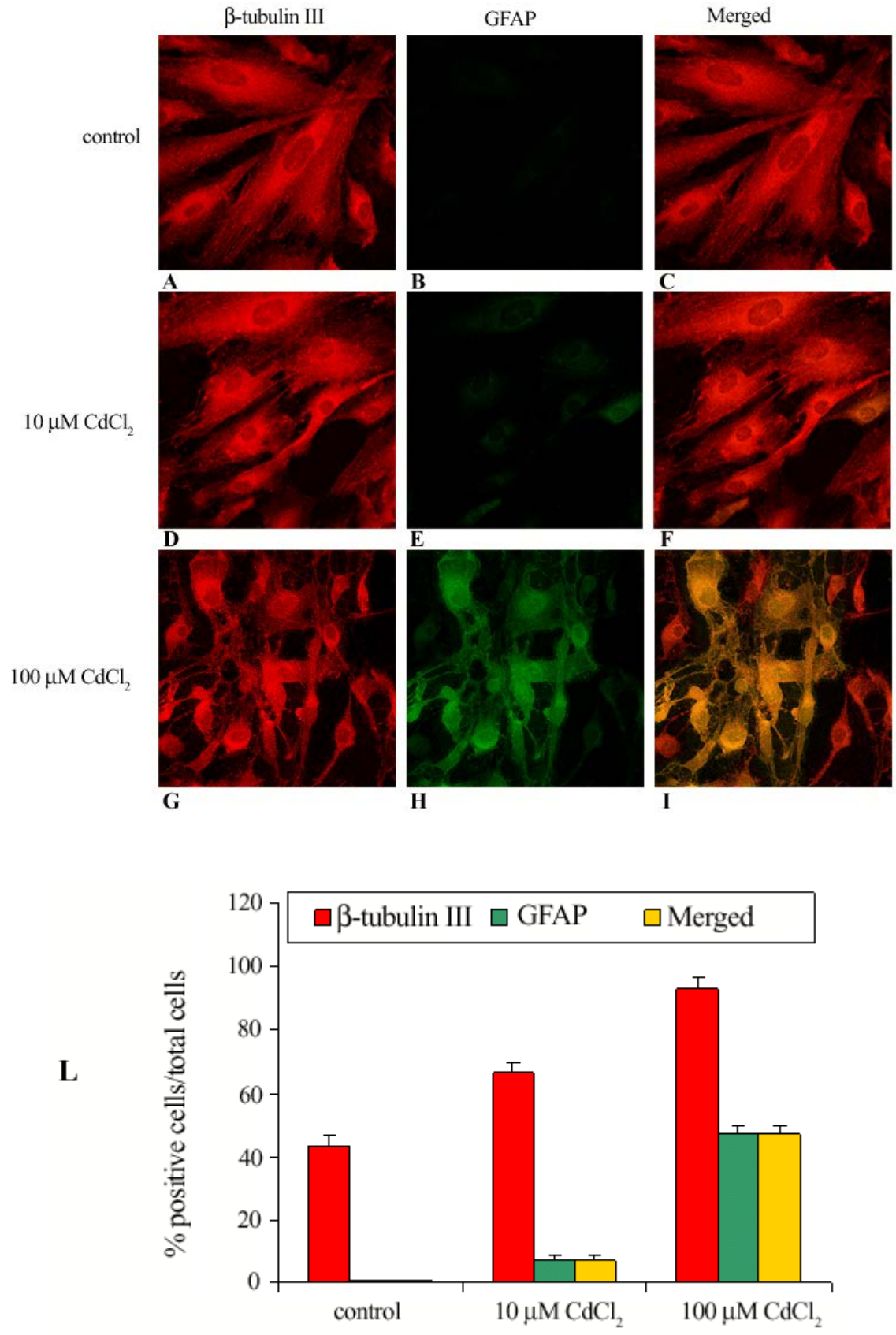


Figure 10. β-Tubulin III and GFAP expression in FNC-B4 neuroblsts before and after stimulation with $CdCl_2$. A, B, C: control, untreated cells. Only cells positive to

β-Tubulin III but no cells positive to GFAP have been detected. Merged image shows no co-localization of β-Tubulin III and GFAP in the same cells. D, E, F: 10 μM $CdCl_2$-treated cells. After the treatment, numerous cells positive to β-Tubulin III and some cells positive to GFAP have been detected. In the merged image the yellow color indicates the co-localization of β-Tubulin III and GFAP in the same cells. G, H, I: 100 μM $CdCl_2$-treated cells. Most of the cells are positive both to β-Tubulin III and to GFAP; co-localization of β-Tubulin III and GFAP is evident in the merged image (yellow colour). Immunohistochemistry and confocal laser scanning microscopy. Total magnification: x630. L: cells were scored as either positive or negative for β-Tubulin III and /or GFAP, and results were expressed as the percentage of positive cells ± SE (calculated by counting the number of stained cells over total in 15 separate fields per slide). In control, only cells positive to β-Tubulin III were detected; the number of cells positive both to β-Tubulin III and to GFAP after stimulation with 10 and 100 μM $CdCl_2$ significantly increased (*p<0.01 *vs.* control cells) with a dose-dependent pathway. *Ordinate:* cells positive to β-Tubulin III and to GFAP (% over total cells); columns are mean ± SE. Results are obtained from three separate experiments (n=3).

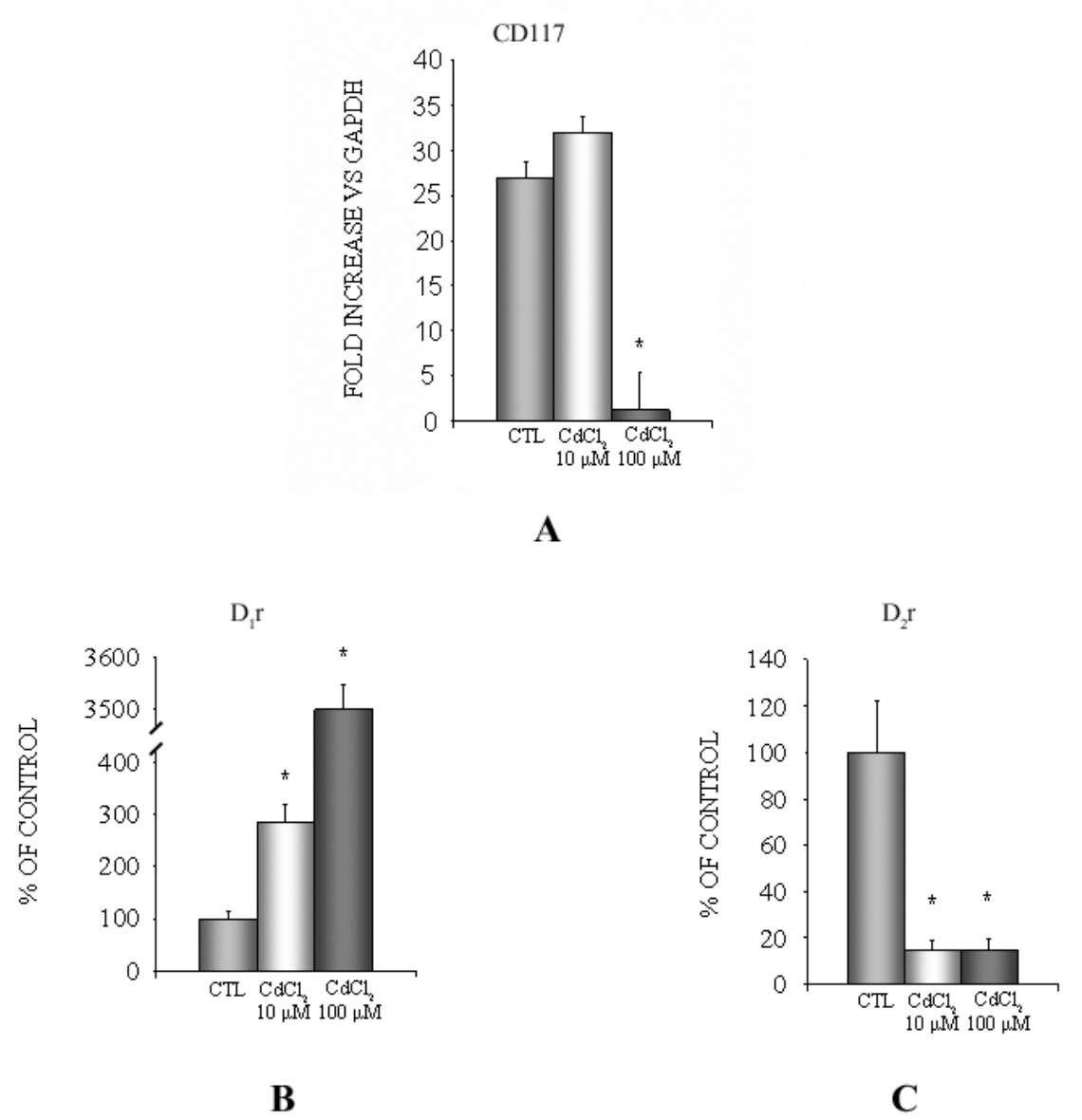


Figure 11. Quantitative gene expression analysis of CD117 and of dopamine D_1 and D_2 receptors in FNC-B4 before and after stimulation with $CdCl_2$. A: mRNA expression of CD117 is significantly decreased after 100 µM $CdCl_2$ treatment in comparison with 10 µM $CdCl_2$ exposure and with control (CTL) cells. B: the exposure of FNC-B4 cells to $CdCl_2$ causes a dose-dependent increase in mRNA expression of dopamine D_1 receptor. C: mRNA expression of dopamine D_2 receptor is decreased in FNC-B4 cells after $CdCl_2$ treatments.

3.3 Cadmium and human foetal spinal cord

Treatment of the human foetal spinal cord specimens for 24 h with $CdCl_2$ 10 and 100 μM induced deep morphological changes in the spinal cord feature (Fig. 12). The ventral horns showed relevant changes both in cell number and distribution after $CdCl_2$ treatment compared to the untreated specimens. When increasing the $CdCl_2$ concentration (10 and 100 μM) the cell number in the ventral horns appeared dramatically decreased and the characteristic aspect of the ventral horns shape deeply modified (Fig. 12). In the dorsal horns of the spinal cord, after the same $CdCl_2$ treatment, a dorsally-directed enlargement of the central canal and a progressive deformation of the dorsal median septum clearly appeared (Fig. 12). After $CdCl_2$ 100 μM treatment the dorsal median septum showed a total overthrow with consequent disarrangement of the spinal cord dorsal region morphology (Fig. 12).

Upregulation of intermediate filament proteins, in particular glial fibrillary acidic protein (GFAP), by reactive astrocytes is perhaps the best known hallmark of reactive astrocytes and reactive gliosis (Pekny and Nillson, 2005). Therefore, GFAP expression after $CdCl_2$ treatment was evaluated by immunohistochemistry and Western blot analysis (Fig. 13). Immunohistochemical study showed an increase in the expression of GFAP

after $CdCl_2$ treatment in comparison to untreated specimens (Fig. 13 A-B). These data were confirmed by Western blot analysis (Fig. 13C).

The expression of β Tubulin III, marker of immature neurons (Gulisano et al., 2009), was also evaluated. The western blot analysis demonstrated a significant decrease of β Tubulin III expression after $CdCl_2$ exposure respect to control specimens (Fig. 14A), indicating a loss of neural cells following the treatment.

The reduction of the neuronal compartment was accompanied by the activation of the apoptotic pathway, as demonstrated by the Western blot analysis of some apoptotic markers. In fact, the exposure of human foetal spinal cord specimens to increasing concentrations of $CdCl_2$ caused an expression level increase of both full-length and cleaved PARP (Fig. 13 D). Furthermore, $CdCl_2$ treatment induced upregulation of Caspase 8 (Fig. 14 C), commonly not expressed in neural precursor cells and required for initiating the apoptotic cascade (Ricci-Vitiani et al., 2004) as well as of NGFRp75 (Fig. 14 B), specifically involved in motor neuron cell death (Pehar et al., 2004).

In order to focus our study on the effects of $CdCl_2$ on the spinal cord ventral horns, the immunohistochemical analysis of ChAT, a specific marker of motor neurons, was performed (Fig. 15 A). The number of ChAT positive neurons was significantly decreased after the treatment with different concentrations of $CdCl_2$ in a dose dependent manner (Fig. 15 B).

According to the decrease of ChAT positive cells, the number of apoptotic cells in the ventral horns, as revealed by TUNEL assay (Fig. 15 C-D), increased in the specimens treated with $CdCl_2$ in comparison to control in a dose dependent manner.

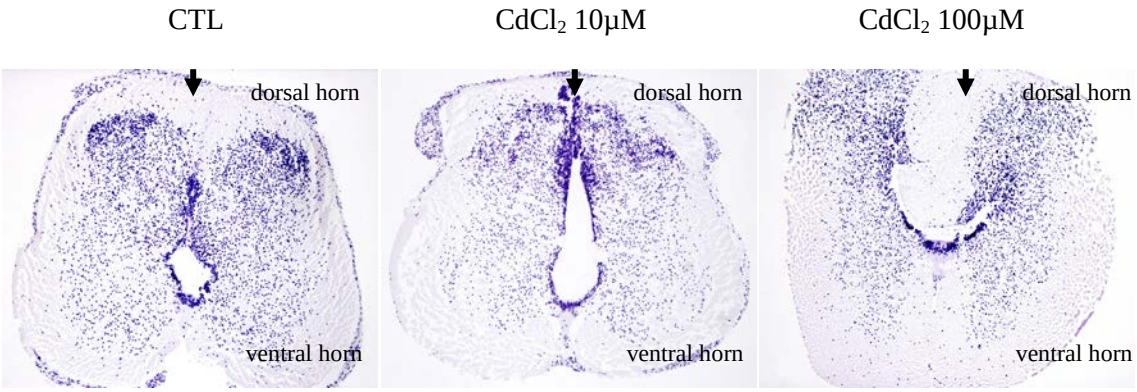


Figure 12. Morphological modifications after treatment with $CdCl_2$ in the lumbar region of spinal cord from a 10-week-old human foetus. Fragments of spinal cord were cultured on organ-culture dishes in serum-free medium for 24 h and then untreated or incubated with $CdCl_2$ (10 and 100 µM). In control specimens (CTL) the typical shape of dorsal and ventral horns was observed; dorsal median septum, ventral median fissure and ependymal canal were correctly formed. After the treatment with 10 µM $CdCl_2$ the cell density in the ventral horns was significantly reduced and the white matter (in light pink) appeared more evident. Dorsally, the median septum (↓), extending towards the dorsal surface of the spinal cord, showed a degenerative aspect. When human foetal spinal cord was treated with 100 µM $CdCl_2$, the decrease of cell density in the ventral horns was evident. In the posterior area, the dorsal median septum, after a total dehiscence, was occupied by a rarefied zone with a scant number of cells and with the aspect of white matter. Haematoxylin Eosin. Total magnification: x40

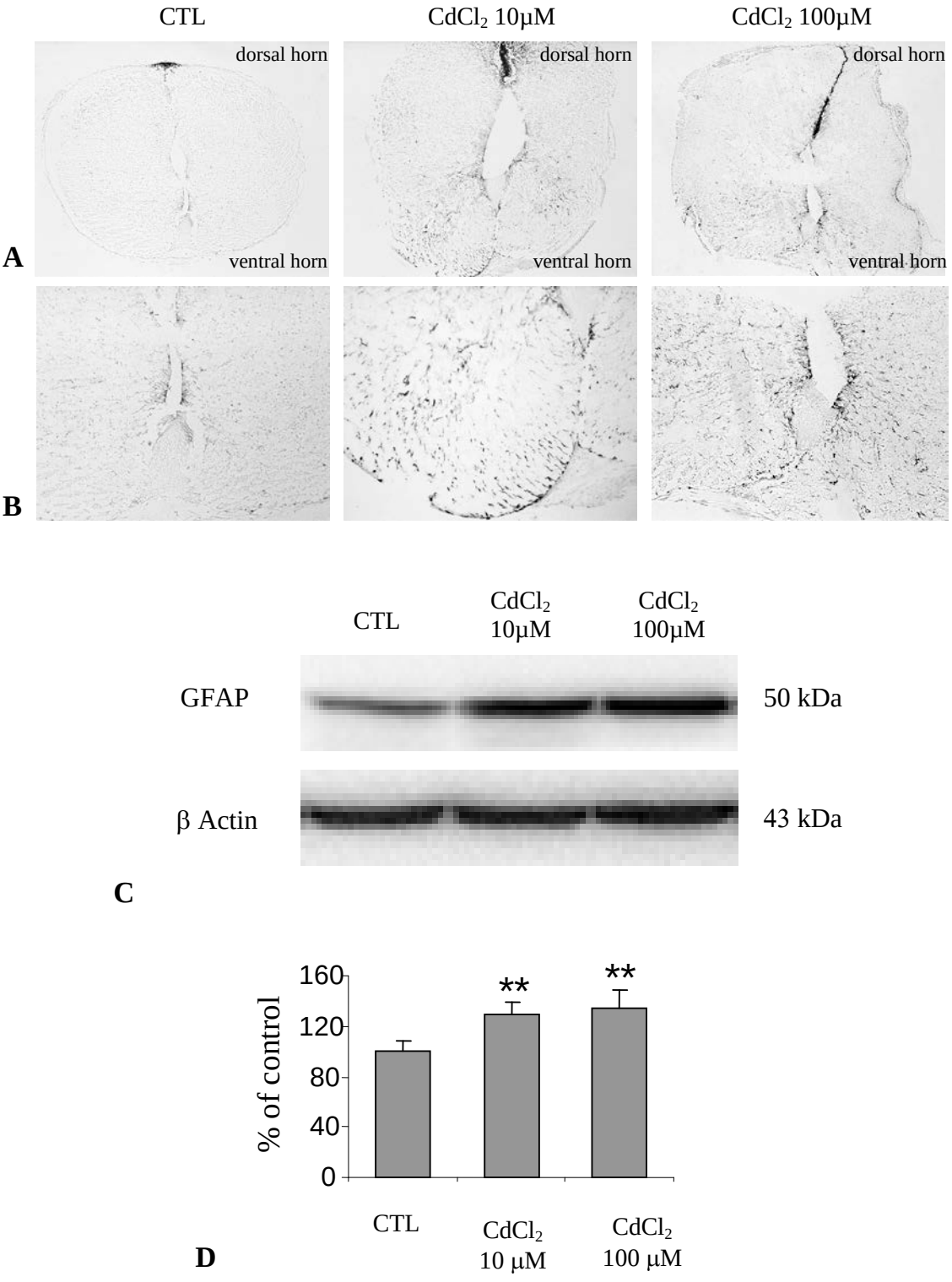


Figure 13. GFAP expression in the lumbar region of human spinal cord from 9-12-week-old fetuses. A: in control specimens only few GFAP positive cells were detected (in black), while the GFAP positivity after stimulation for 24h with 10 and 100 µM $CdCl_2$ was significantly increased. Total magnification x40. B: particular of the ventral horns, total magnification x100. C: western blot analysis of total

lysates from spinal cord untreated (CTL) or following 24h of incubation with $CdCl_2$. Western blot analysis with anti-GFAP antibody revealed a single band migrating approximately at the expected 50 kDa molecular mass. Note the increased expression of GFAP after $CdCl_2$ treatment. D: the densitometric quantification of the bands, expressed as percentage over control (taken as 100%), confirmed the increased GFAP expression following 24h $CdCl_2$ exposure. β actin signal was used for the normalization of GFAP protein expression. Data were obtained from three separate experiments (P<0.01 $CdCl_2$ 10 and 100 µM vs CTL).**

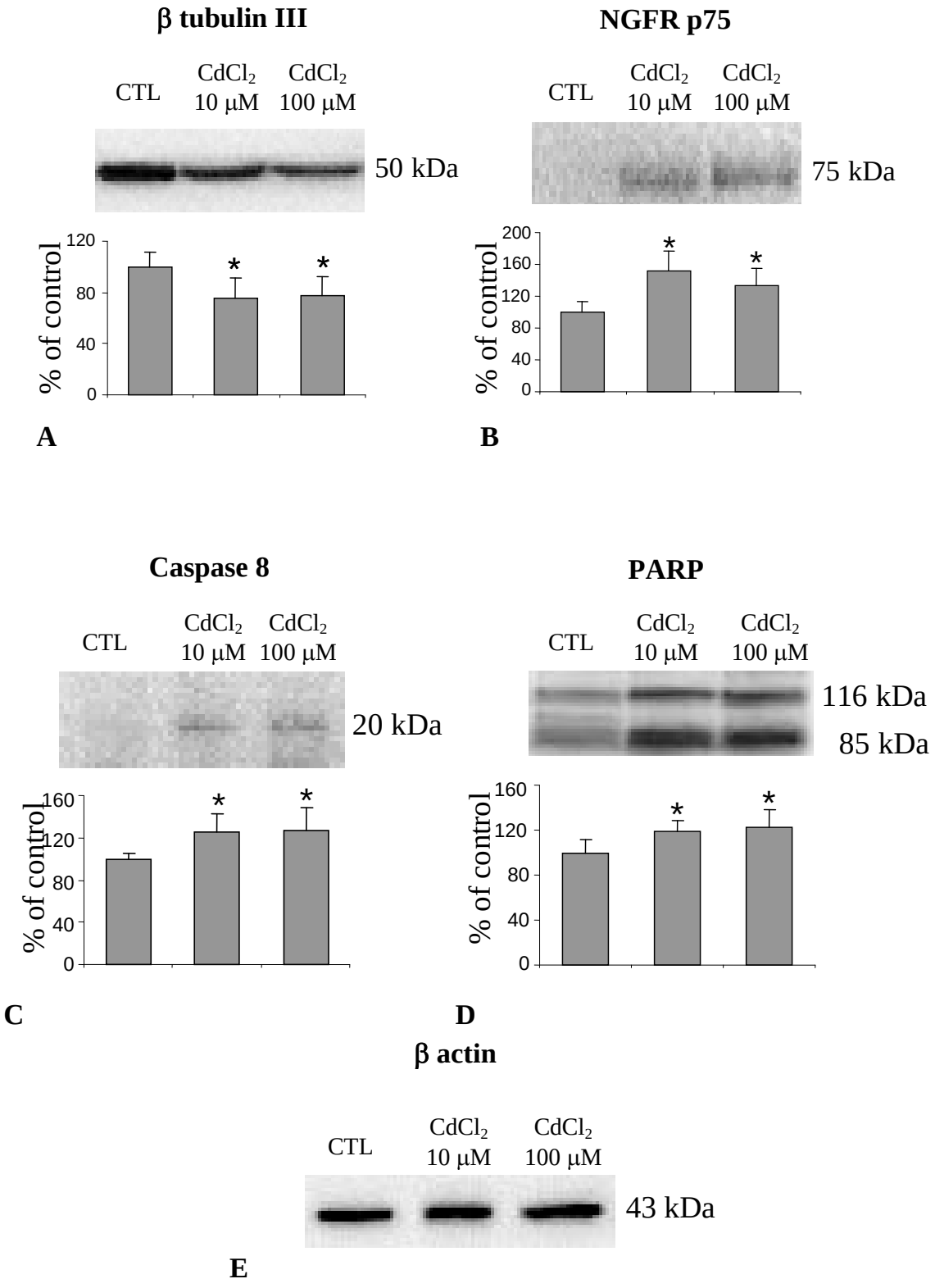
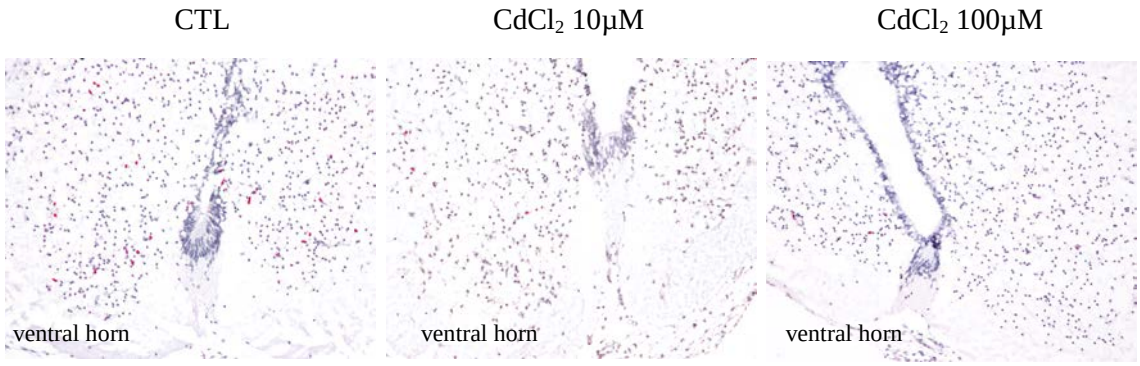
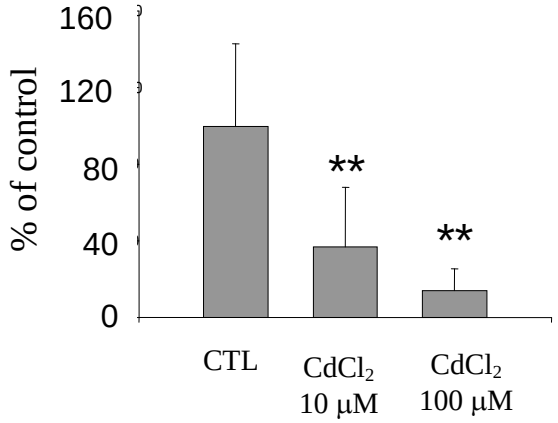


Figure 14. Western blot analysis of spinal cord protein extract stained with antisera to β Tubulin III, NGFRp75, PARP or caspase 8. Western blot analysis of spinal cord samples from 9-12-week-old human fetuses showed a significant decreased expression of the neuronal marker β Tubulin III following 24h $CdCl_2$

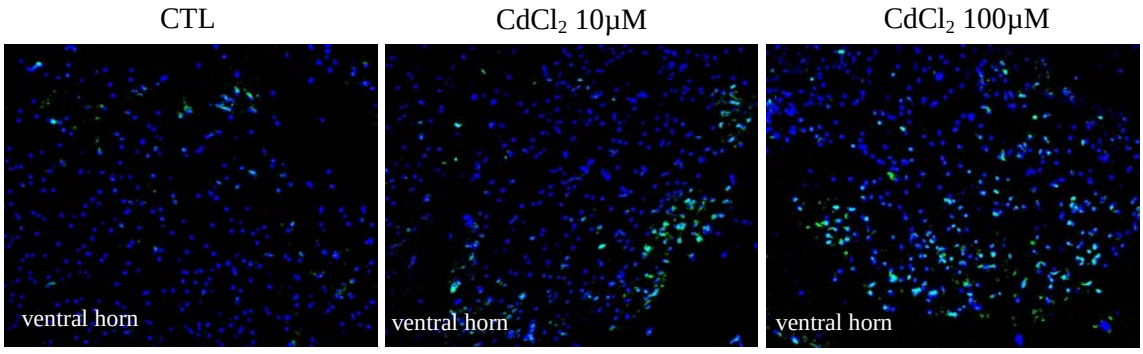
exposure, indicating a neuronal loss after the treatment (panel A). These data were confirmed by the analysis of some apoptotic markers (NGFRp75, PARP and the cleaved form of caspase 8) that resulted upregulated after $CdCl_2$ exposure. The quantification of bands corresponding to the proteins examined by Western blot was made directly on the films by Quantity One Software and was expressed as percentage over control (taken as 100%) (panels B, C, D). β actin signal was used for the normalization of each protein expression (panel E). Data were obtained from three separate experiments (*P<0.05 $CdCl_2$ 10 and 100 µM vs CTL).


CTL
CdCl2 10µM
CdCl2 100µM
ventral horn
ventral horn
ventral horn

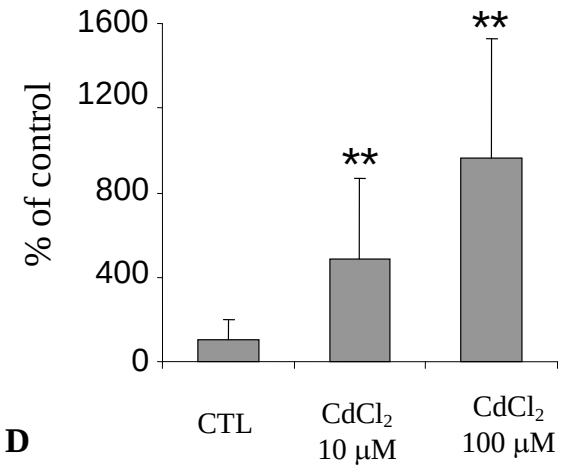
A


160
120
80
40
0
% of control
**
**
CTL
CdCl2 10 µM
CdCl2 100 µM

B


CTL
CdCl2 10µM
CdCl2 100µM
ventral horn
ventral horn
ventral horn

C



D

Figure 15. Immunohistochemical analysis of ChAT and TUNEL assay in the ventral horns of spinal cord samples from 9-12-week-old human fetuses. A: cross sections (conducted at lumbar level) of human foetal spinal cord were immunostained with anti-ChAT, a specific marker of motor neurons (red positivity). Cell nuclei were counterstained with Haematoxylin. Total magnification x100. B: the number of ChAT positive cells in control and treated specimens was counted and expressed as percentage over control (taken as 100%). ChAT positive motor neurons significantly decreased in the specimens treated with $CdCl_2$ 10 and 100 μM in comparison to control specimens, in a dose dependent manner. Data were obtained counting 15 separate fields for each slide of three independent experiments (P<0.01 $CdCl_2$ 10 and 100 μM vs CTL; P<0.05 $CdCl_2$ 100 μM vs $CdCl_2$ 10 μM). C. TUNEL (dUTP nick-end labeling) for the staining of apoptotic cells was performed on cross sections of human foetal spinal cord at lumbar level. Cell nuclei were counterstained with DAPI (blue); the nuclei of apoptotic cells were stained with green fluorescence. The merged cyan colour identified the apoptotic cells above the number of total cells. D: the number of cyan cells in control and treated specimens was counted and expressed as percentage over control (taken as 100%). TUNEL positive cells significantly increased after $CdCl_2$ treatment in comparison to control untreated specimens in a dose dependent manner. Data were obtained counting 15 separate fields for each slide of three independent experiments (**P<0.01 $CdCl_2$ 10 and 100 μM vs CTL; P<0.05 $CdCl_2$ 100 μM vs $CdCl_2$ 10 μM).**

4 CONCLUSIONS

4.1 Cadmium and normal/tumour human breast cell lines (MCF-7 and MCF-10A respectively)

The analysis of the biological effects of cadmium on normal and tumor breast cell lines (MCF-7 and MCF-10A respectively) shows that $CdCl_2$ exposure affects the morphology and the proliferation both of MCF-7 and MCF-10A cells. The 10 μM $CdCl_2$ exposure reveals a modifications in size, shape a growth of some of MCF-7 and MCF-10A cells in comparison to control, untreated cells. Some of the cells are distributed as small groups or separately. They appear smaller than the untreated cells and their shape changes to spherical morphology. These alterations are more evident in MCF-10A in comparison to MCF-7. The 100 μM $CdCl_2$ exposure reveals deep modifications in both the cell lines. Only few separate cells are observable; they appear smaller than untreated cells and with spherical morphology. The 10, 100 μM and 1 mM $CdCl_2$ exposures inhibits significantly both MCF-7 and MCF-10A cell proliferation in a dose-dependent manner. Moreover 1 μM $CdCl_2$ exposure don't affect the PARP-mediated levels of apoptosis in MCF-7 cells but increases significantly the apoptosis in MCF-10A cells in comparison to control untreated cells. 10 and 100 μM $CdCl_2$ exposure increases significantly the PARP-mediated apoptosis in MCF-10A cells in a dose-dependent manner.

The 10 μM $CdCl_2$ exposure increases significantly also the PARP-mediated apoptosis of MCF-7 cells whereas the 100 μM $CdCl_2$ exposure shows a decreases of levels of apoptosis probably because of the presence of a cadmium precipitate that don't allow the metals to fully affect the cells. These results emphasize that MCF-10A cells are more sensitive than MCF-7 to $CdCl_2$ exposure toxic effects.

Angiogenic potential evaluated using the CAM assay show that MCF-7 cells present a strong angiogenic response due to their typical tumor characteristics. Moreover, in MCF-10A cells, 10 μM $CdCl_2$ exposure induces an angiogenic response similar to that induced by untreated MCF-7 cells; these increasing of the angiogenic potential of MCF-1oA after $CDCl_2$ exposure is associated to a parallel increasing of VEGF levels as evaluated by western blot analysis.

4.2 Cadmium and primary human neuroblast cell line FNC-B4

This investigation of the biological effects of cadmium on primary human neuroblast cell line FNC-B5 provides the first evidence that $CdCl_2$ is simultaneously able to strongly inhibit the growth of developing neurons (FNC-B4) and induce differentiation of neural progenitor cells into distinct neuronal and glial cell lineages.

Environmental pollutants and transition metals such as cadmium have short-term and long-term effects on neuron development. Some studies demonstrate that cadmium may disturb the natural oxidation/reduction balance in cells through various mechanisms, which interferes with cellular signaling and gene expression systems.

In fact, in some cell lines such as human neuroblastoma NB-1 cells, exposure to $CdCl_2$ induces a significant neurite outgrowth, suggesting a role in neuron differentiation for cadmium). Furthermore, administration for 3 h of 100 or 150 μM $CdCl_2$ inhibits proliferation and induces several functional differentiation markers in some cell lines.

The results of this study show that the treatment of human immature neuron FNC-B4 cells, with different concentrations of $CdCl_2$ significantly affects cell growth. Indeed, low concentrations of $CdCl_2$ stimulates cell growth, whereas higher concentrations causes a decrease in cell proliferation. The latter effect, according to previous study, is associated to morphological and biochemical alterations as well as to induction of apoptotic cell death consistent with the induction of cell differentiation toward glial/neural precursor.

Low concentrations of $CdCl_2$ induces the appearance of a proliferative phenotype in FNC-B4 cells associated with an increase in CD117 gene expression, a marker which is associated with cellular proliferative potential. The exposure of FNC-B4 neuroblasts to high concentrations of

$CdCl_2$ induced significant morphological and cytoskeletal alterations, remarkable reduction of proliferation and of CD117 gene expression. Several cells also show signs of apoptosis as confirmed by morphological observation and by the increased level of cleaved PARP. These findings suggest that $CdCl_2$, depending on its concentration, is able to change and thus regulate distinct biological responses. The exposure of FNC-B4 to increasing concentrations of $CdCl_2$, induces a significant decrease in the expression of some neural markers such as Nanog, Nestin and CD15. This latter gene distinguishes highly proliferative cells and it is strongly expressed in neuronal regions with prolonged neurogenesis *i.e.* the olfactory epithelium. On the other hand, neural/glial differentiation markers such as GFAP and β-Tubulin III became robust. Moreover, the present study shows that high concentration of $CdCl_2$ caused a significant increase of the marker, CD133. CD133 is a plasma membrane marker found in several types of somatic stem cells, including neural stem cells; however, the expression of CD133 is not limited to stem progenitor cells but it also occurs both in adult and in differentiated cells. Irrespective of cell type, CD133 is specifically associated with plasma membrane protrusions and with the alterations of cell surface deeply involved in neuronal differentiation. In agreement the aforementioned studies, this study suggests that the treatment with $CdCl_2$ forces FNC-B4 cells to change from immature cells to more differentiated cells, expressing both glial and neural

markers. We think that CD133 may be considered as a new marker to define more differentiated progenitor cell *in vitro*.

Recent studies suggest that dopamine may play a crucial role as a modulator in olfactory discrimination and in olfactory processing as well as in several disorders with olfactory deficits, such as Parkinson disease, characterized by altered dopamine homeostasis in olfaction-related brain regions. However, dopamine is present in the brain early in development and functional dopamine receptors are expressed in the central nervous system prior to the onset of synaptogenesis suggesting a role for dopamine in brain development that may be independent of its role at the synapse in the mature central nervous system. Neurotransmitters such as dopamine can influence brain development by modulating neurogenesis or neuronal and glial cell differentiation. Recent studies show that dopamine receptor activation influences the cell cycle of neuroepithelial cells in the lateral ganglionic eminence and in the cerebral wall. The findings of this study demonstrate that $CdCl_2$ simultaneously up-regulates dopamine D_1 receptor expression and down-regulates dopamine D_2 receptor expression. In particular, increasing $CdCl_2$ concentrations induces a progressive increase in dopamine D_1 receptor expression, while low and high $CdCl_2$ concentrations caused a significant decrease in dopamine D_2 receptor expression. Since dopamine D_1 and D_2 receptor activation produces opposite effects on precursor cell activity, we can hypothesize that

dopamine's overall effects correlate with relative numbers and activity of each receptor subtype expressed on the FNC-B4- neuroblast cell line.

During development of the nervous system, neural stem cells give rise to both the neuronal and glial population. A dominant model of neural development is that neuronal and glial lineages diverge early, with neuroepithelial precursor giving rise to neuron-restricted and glial-restricted progenitors. Although a set of molecular markers have been defined for neural progenitors, their identification, characterization and function in neuronal and glial lineages have generated much discussion and are still not fully understood. Recent studies have demonstrated that some radial glia, the first cells differentiated within the embryonic neuroepithelium, have stem cell characteristics and produce neurons and astrocytes. In the adult brain, neural stem cells are found in two regions of the periventricular germinal matrix, the subventricular or subependymal zone of the lateral ventricles and the subgranular zone of the hippocampal dentate gyrus. In addition to the “neurogenic” regions of the brain, multipotential neural stem cells are isolated from postnatal cerebellum. These cells express markers associated with neural progenitor and stem cells as well as GFAP, a defining marker of astroglia. GFAP-positive cells, with characteristics of neural stem cells are obtained from fetal human brain parenchyma. These cells co-express both glial and neuronal markers,

and they are able to differentiate into distinct neuronal and glial cell lineages.

From the data of this study emerge that FNC-B4 neuroblasts treated with high concentration of $CdCl_2$ are forced to differentiate toward cells expressing both neuronal (β-Tubulin III) and glial (GFAP) markers; this neuronal/glial precursor still expresses vimentin, even though differently arranged in comparison to control cell and to neuroblasts treated with low concentration of CdCl2, suggesting the possibility to evolve both toward neuron or toward glial cells. As a matter of fact, vimentin is a critical cytoskeletal protein, involved in oxidative stress response and initially expressed by nearly all neuronal precursor but substituted by neurofilaments in post mitotic neurons; the presence of vimentin in post mitotic cells is considered a typical marker of glial cells.

A remarkable feature of olfactory neurons is that they have a half-life in the range of weeks and are replaced by new neurons that differentiate from a progenitor present in the olfactory epithelium. Because FNC-B4 cell exposure to $CdCl_2$ induces a decrease in some neural markers and an increase in neural differentiation marker expression we can hypothesize that $CdCl_2$ might represent a specific signal for olfactory lineage differentiation. We can also speculate that $CdCl_2$ in FNC-B4 neuroblasts might represent a selective cue by which, in a heterogeneous degree of maturity of the primary cell culture, the more committed progenitors and/or

differentiated mature neurons rapidly move towards apoptosis, whereas the undifferentiated neural precursors are positively selected and forced to a state of active differentiation into glial cells and/or neurons, via an intermediate precursor expressing both glial and neuronal markers. The identification of the neuronal/glial cell precursor, the signaling that regulates self-renewal and differentiation of FNC-B4 cells, is basic and crucial for understanding the mechanisms of pathological processes leading to neurological diseases.

4.3 Cadmium and human foetal spinal cord

It is well established that multiple gene-environmental interactions which govern different biochemical pathways are linked to the etiopathogenesis of neurodegenerative diseases. Among the environmental agents linked to neurodegenerative diseases, pollutants deriving from industries certainly play a crucial role. Although indications exist that industrial chemicals can cause damages on central nervous system during its development, only 5 of 80,000 known chemicals have been identified as toxic to human neurodevelopment. The heavy metals such as Cd are certainly likely candidates because of their proven neurotoxicity, ubiquitous nature and causal epidemiological link with neuropsychological disorders and neurodegenerative diseases.

The neurotoxic effects of Cd have been reported in neonatal mouse brain and young rat brain but only few data are available about the role of Cd on human developing central nervous system. In humans, occupational exposure to Cd is associated with neuropsychological disorders, and Cd exposure is reported to be a possible cause of ALS; Cd is shown to selectively damage striatum, and Parkinsonism has been reported in a 64-year-old man exposed to Cd at a high dose. Cd-induced damages on CNS may be explained by the Cd ability of activating apoptotic pathways.

Recently, several reports have shown that cadmium can induce apoptosis of many tissues and cells both *in vivo* and *in vitro*, such as the cells of the respiratory system, the testis, the kidney, the liver, and the immune system. This evidence indicates that apoptosis probably plays a very important role in acute and chronic intoxication by Cd.

In human lung epithelial fibroblast cell line, Cd has been shown to cause apoptosis by the Caspase-8-dependent Bid cleavage, activation of Caspase-9 and -3, and PARP cleavage. In human lymphoma cells it has been demonstrated that Caspase 8 may be the most apical Caspase induced by Cd in the apoptotic Caspase-dependent pathway; furthermore, a recent research has demonstrated that the activation of NGFRp75 induced motor neuron apoptosis in rat embryonic spinal cord.

Besides the activation of different apoptotic pathways, recent researches have demonstrated that astrocytes play a complex role in repair after spinal

cord injury. The vicinity of a traumatic injury, environmental cues associated with cell damage and neuroinflammation induces astrocytes to proliferate, migrate, differentiate and form a dense network bordering the lesion site. This response not only contributes to the formation of the glial scar but it also provides support and guidance for axonal growth and aid in improving functional recovery after spinal cord injury.

Several studies using GFAP-deficient mice demonstrated that animals lacking GFAP have increased hippocampal degeneration after brain injury , form an abnormal glial scar after brain and spinal cord lesions, produce astrocytes with impaired migratory abilities *in vitro*, and are more susceptible to death after cervical spinal cord injury.

Reactive astrocytes can arise either from astrocytes that are already present at the time of injury, or from progenitor cells that are found either in regions surrounding the central canal or the subpial region of the spinal cord.

This study focused on the effects of Cd on human spinal cord at early stages of development, when its vulnerability to neurotoxicants is high. Spinal cord slices were used *in vitro*: an experimental model in which the tissue cytoarchitecture and extracellular matrix connections are preserved, and the interactions between neuronal and glial cells are analogues to those *in vivo*.

This study provided the first evidence that Cd is able to affect two different cell populations in developing human spinal cord; in fact, important signs of apoptosis are evident in motor neurons and, at the same time, a significant gliosis arises.

The findings of this study indicate that cleavage of Caspase 8, NGFRp75, and PARP with consequent specifically reduction of the motor neurol compartment. Cd-induced apoptosis is associated to a significant gliosis as demonstrated by a significant increase of GFAP expression. The number of GFAP positive cells increased after Cd treatment surrounding the area of motor neuron loss. As well as in the proximity of the ependymal canal. The significance of this increased GFAP expression is not yet clear. It is possible that Cd may activate astrocytes, thus playing a role in the organizational events of glial scar formation. This increase in GFAP expression may also be related to the attempt of glial cells to be supportive of recovery. Motor neurons death and consequent glial activation observed in this study after Cd treatment, may be explained considering the molecular mechanism of Cd. In fact, Cd ion easily substitutes for the Calcium and Zinc ions in several biological systems because it carries the same charge and it has a similar radius. For its ability in substituting and/or displacing Calcium and Zinc, Cd is involved in the generation of reactive oxygen species, inhibition of DNA repair enzymes, deregulation of cell proliferation as well as DNA methylation.

Disregulation of calcium and abnormal Calcium homeostasis are decisive in long-term neurodegeneration diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and ALS. During CNS development, when the permeability of BBB is changing, Cd, interfering with biochemical events where Calcium or Zinc are involved, might deeply affect the developing of nervous structures.

Most existing data on acute and chronic developmental neurotoxic effects have been obtained in animal studies, and only a few studies have specifically dealt with human developmental neurotoxicity.

This study provided the first evidence that Cd is able to induce both a decrease in β Tubulin III and ChAT expression and an increase in GFAP and apoptotic markers expression during human spinal cord development.

Apoptosis plays an important role during embryonic development and tissue homeostasis. This complex process is particularly important in the development of the central nervous system, involving an interplay between intrinsic cellular programs and extrinsic cues such as growth factors.

Our results in human spinal cord showed that the exposure to Cd caused an increase in NGFRp75 expression. Our observations also indicate that Cd can induce increased number of apoptotic cells such as motor neurons. We therefore speculated that Cd-induced neurotoxicity can be caused by impaired neurogenesis, resulting in markedly reduced neuronal compartment. In this study we also showed that Cd exposure is associated

to a significant gliosis as demonstrated by the increase of GFAP expression. Recent researches have demonstrated that astrocytes play a complex role in repair after spinal cord injury. The vicinity of a traumatic injury, environmental cues associated with cell damage and neuroinflammation induce astrocytes to undergo hypertrophy, proliferate, migrate, differentiate, and form a dense network bordering the lesion site.

Our results in human spinal cord are in agreement with those of the aforementioned studies. In fact, the number of GFAP positive cells increased after Cd treatment not only in the ventral horns in the vicinity of motor neuron loss, but also in the proximity of the ependymal canal. The significance of this increased GFAP expression is not yet clear. It is possible that Cd may activate astrocyte, thus playing a role in the organizational events of glial scar formation. This increase in GFAP expression may also be related to the attempt of glial cells to be supportive of recovery.

In conclusion, our study extends to a human model previous observations in animal models on the dual role of Cd in neural tissue both stimulating glial activation and neuronal death. In addition, the present results provide the first evidence that during the human development Cd does not deserve just a neurotoxic role but also a central role in controlling the fate of glial cells, through the regulation of astrocytes. This findings shed new light on the possible link between chemicals and neurodevelopmental disorders.

In this study we demonstrate that Cd treatment induces an increase of apoptotic markers expression (NGFRp75, caspase 8 and PARP) in human foetal spinal cord. Specific markers for motor neurons appear decreased after Cd exposure confirming that apoptosis specifically affects motor neurons of the ventral horns; Cd-induced apoptosis is associated to a significant gliosis as demonstrated by a significant increase of GFAP expression. Cd exposure significantly affects the ratio motor neurons/glial cells in human spinal cord at early stages of development; the decreased number of motor neuron as well as the increased of gliosis observed in the ventral horns may deeply affect function and development of human foetal spinal cord. Motor neurons death as well as proliferation of glial cells observed in this study after Cd treatment, may be explained considering the molecular mechanism of Cd. In fact, Cd ion easily substitutes for the Calcium and Zinc ions in several biological systems because it carries the same charge and it has a similar radius. For its ability in substituting and/or displacing Calcium and Zinc, Cd is involved in the generation of reactive oxygen species, inhibition of DNA repair enzymes, deregulation of cell proliferation as well as DNA methylation.

Disregulation of Calcium and abnormal Calcium homeostasis are decisive in long-term neurodegeneration diseases such as Alzheimer's disease, Parkinsons's disease, Huntington's disease, and ALS. During CNS development, when the permeability of BBB is changing, Cd, interfering

with biochemical events where Calcium or Zinc are involved, may deeply affect the developing of nervous structures.

5 DISCUSSION

Cadmium (group IIB of the periodic table of elements) is a heavy metal posing severe risks to human health. Up to this day, it could not be shown that cadmium has any physiological function within the human body. Interest has therefore risen in its biohazardous potential.

Cadmium is regularly found in ores together with zinc, copper and lead. Therefore volcanic activity is one natural reason for a temporary increase in environmental cadmium concentrations. Cadmium is widely used in industrial processes, e.g.: as an anticorrosive agent, as a stabilizer in PVC products, as a colour pigment, a neutron-absorber in nuclear power plants, and in the fabrication of nickel-cadmium batteries. Phosphate fertilizers also show a big cadmium load. Although some cadmium-containing products can be recycled, a large share of the general cadmium pollution is caused by dumping and incinerating cadmium-polluted waste. In Scandinavia for example, cadmium concentration in agricultural soil increases by 0.2% per year.

Principally there are two possible ways of cadmium resorption: gastrointestinal and pulmonary. The uptake through the human

gastrointestinal is approximately 5% of an ingested amount of cadmium, depending on the exact dose and nutritional composition. Several factors can increase this amount, such as low intakes of vitamin D, calcium, and trace elements like zinc and copper. Concerning zinc and calcium, it is assumed that their molecular homology could be a reason for a compensatory higher cadmium resorption. Furthermore a high fiber diet increases the dietary cadmium intake. The most important metabolic parameter for cadmium uptake is a person's possible lack of iron. People with low iron supplies showed a 6% higher uptake of cadmium than those with a balanced iron stock. This is the main reason for the higher cadmium resorption in people with anaemia and habitual iron deficit, such as children or menstruating women. Low iron blood levels stimulate the expression of DCT-1, a metal ion transporter in the GI tract, serving as a gate for cadmium resorption. The major source of inhalative cadmium intoxication is cigarette smoke. The human lung resorbes 40–60% of the cadmium in tobacco smoke. Smokers generally have cadmium blood levels 4–5 times those of non-smokers. Workers exposed to cadmium-containing fumes have been reported to develop acute respiratory distress syndromes (ARDS). Once taken up by the blood, the majority of cadmium is transported bound to proteins, such as Albumin and Metallothionein.

The first organ reached after uptake into the GI-blood is the liver. Here cadmium induces the production of Metallothionein. After consecutive

hepatocyte necrosis and apoptosis, Cd-Metallothionein complexes are washed into sinusoidal blood. From here, parts of the absorbed cadmium enter the entero-hepatical cycle via secretion into the biliary tract in form of Cadmium-Glutathione conjugates. Enzymatically degraded to cadmium-cysteine complexes in the biliary tree, cadmium re-enters the small intestines. The cadmium concentration increases in the renal cortex, retina and other body tissues with age. There is some proof that cadmium can cause cancer. Some publications suggested an association of cadmium and renal cancer in humans. Consequently, the IARC (International Agency for Research on Cancer) decided to classify cadmium as a human carcinogen group I. Latest data however supports the assumption that only an uptake of cadmium via the respiratory system has carcinogenic potential.

This research provides confirmations about the potential risk for human health caused by cadmium exposure. More studies are necessary to elucidate the mechanisms at the basis of cadmium toxicity.

At present our research on the biological effects of cadmium on different cells, tissues and organs experimental models adopt primary human neurons from foetal basal nuclei as experimental model.

Thanks to a collaboration with an English research group another experimental model recently adopted includes the study of cadmium exposure on primary (murine) retinal ganglion neurons. The evaluation of

the morphology, cell proliferation and angiogenic potential after $CdCl_2$ exposure performed by our research group is integrated by electrophysiological analyses on ion channels performed by the English scientific group.

For this reason this PhD thesis was written in English language.

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