1.Introduction

1.1 Treatment of colo-rectal cancer: relevance of oxaliplatin in chemotherapy

Colo-rectal cancer, the third most common cancer in both men and women in the world, is currently a public health priority, because it is the second leading cause of cancer deaths in Western countries (American Cancer Society. Cancer Facts & Figures 2009). In the United States alone, 51370 deaths from colorectal cancer were estimated to occur in 2010, which accounted for almost 9% of all cancer deaths; approximately 140000 new cases of colorectal cancer were expected for that same year (Jenal et a., 2009). Nearly 80% of patients will be diagnosed at a stage in which surgery is the primary therapeutic modality. Despite the curative potential of surgical resection, the 5-year survival rates for patients with stage II and III colon cancer (approximately 80% and 60%, respectively) attest to the fact that a significant proportion of patients are destined to have recurrences and to die of this disease (Hayat et al., 2007). Recurrences are also seen in nearly 25% of patients with rectal cancer treated with neoadjuvant or adjuvant therapies and surgery (Sauer , et al., 2004). Between the late 1950s and early 1990s, fluorouracil (5-FU) remained the only chemotherapeutic agent with clinically significant activity against metastatic colorectal cancer. Despite the fact that 5-FU-based chemotherapy may increase the survival and improve the quality of life of patients with advanced colorectal cancer, in comparison with observation (Scheithauer et al., 1993) treatment with 5-FU had reached a plateau of efficacy. During the past 15 years, the introduction of irinotecan and oxaliplatin has progressively changed the management of patients with advanced colorectal cancer. Combination regimes of oxaliplatin and infusional 5-FU/leucovorin (FOLFOX) or capecitabine (XELOX) have emerged as important options in the palliative and adjuvant treatment of colorectal cancer (Grothey and Goldberg, 2004). Adjuvant chemotherapy has improved the prognosis and is curative in some patients with resected colon cancer; 5-FU decreases the risk of cancer recurrence or death by approximately 30%, and the addition of oxaliplatin provides an additional 23% relative risk reduction (Gill et al., 2004). The clinical development of oxaliplatin-based regimens started in the 1990s and along the years, several different combinations of 5-FU, leucovorin, and oxaliplatin, collectively named FOLFOX, have been studied. The

French group led by de Gramont initially found that the combination known as FOLFOX2 produced a response rate of 46% among 46 patients in whom prior treatment with 5-FU and leucovorin had failed (de Gramont et al., 1997). A regimen with a lower dose of oxaliplatin twice monthly (85 mg/m^2) was tested in a phase II trial, in an attempt to reduce the rate of neuropathy seen among patients treated with FOLFOX2; unfortunately, the response rate with FOLFOX3 was only 20% (Andre et al., 1998; Andrè et al., 1999). In one of the trials, a regimen administered twice a month also was tested; such a regimen, which consisted of oxaliplatin (85 mg/m^2 on day 1), leucovorin (200 mg/m^2 in a 2-hour infusion on days 1 and 2), and 5-FU (400 mg/m^2 bolus and 600 mg/m^2 by a 22-hour infusion on days 1 and 2) has became known as FOLFOX4. The response rate was 23.5% among 57 patients who had previously progressed on the same 5-FU and leucovorin regimen. More recently, FOLFOX6 and its modified version (mFOLFOX6) have gained widespread acceptance in the oncology community (Allegra et al., 2009; Tournigand et al., 2004). In mFOLFOX6, leucovorin is administered on day 1 (400 mg/m^2), 5-FU is given as a bolus (400 mg/m^2) on day 1 and as a continuous infusion over 46 hours (2400 mg/m^2), and oxaliplatin is administered as in FOLFOX4 (85 mg/m^2 on day 1).

The FOLFOX family of regimens, already used in Europe, was eventually accepted as one of the standard first-line options for advanced colorectal cancer in the United States after the N9741 trial, which demonstrated the superiority of FOLFOX4 over 5-FU and leucovorin and over 5-FU and leucovorin "bolus" plus irinotecan (Goldber et al 2004, de Gramont et al., 2000). Moreover, oxaliplatin has become an important component of neoadjuvant therapy in patients with resectable or initially unresectable metastatic liver lesions from colorectal cancer (Nordlinger et al., 2008). A large international phase III clinical trial was thus launched to determine whether the improved efficacy observed with oxaliplatin in the metastatic setting translates into benefits in earlier stages (II and III) of colon cancer. In this study, named MOSAIC, the adjuvant use of FOLFOX4 improved 3-year disease-free survival (DFS), compared with 5-FU and leucovorin alone (78.2% vs. 72.9%; hazard ratio [HR] 0.77; P = .002). In the MOSAIC trial, 75% of patients in the group given FOLFOX4 received the planned 12 cycles, and 80.5% of the planned dose of oxaliplatin was actually given (Andre et al., 2004). The final results of this study confirmed that the addition of

oxaliplatin to the 5-FU and leucovorin backbone significantly improved the 5-year DFS (73.3% vs. 67.4%; HR 0.80; *P* = .003) and 6-year overall survival (78.5% vs. 76.0%; HR 0.84; *P* = .023) in the adjuvant treatment of stage II or III colon cancer (Andre et al., 2009). The results of the MOSAIC study have been consolidated by those of a phase III trial conducted by the NSABP (National Surgical Adjuvant Breast and Bowel Project), in which the FOLFOX regimen proved superior to a bolus 5-FU/leucovorin regimen in stage II and III colon cancer (Keubler et al., 2007). Thus, oxaliplatin-containing regimens should be strongly considered after surgery for patients with stage III disease, in whom a significant difference in overall survival was found in subgroup analysis (Andre et al., 2009).

Oxaliplatin represents an important chemotherapeutic agent for the treatment of various other types of gastrointestinal carcinomas (Hoff et al., 2003). Even without a package insert indication in the United States, oxaliplatin is currently indicated as preoperative and definitive treatment in localized esophageal carcinoma according to the guidelines from the National Comprehensive Cancer Network (NCCN Oncology Practice Guidelines in Oncology. Esophageal Cancer version 2-2009.)

Finally, the addition of oxaliplatin to a regimen that contains irinotecan and 5-FU has led to improved survival in advanced pancreatic cancer in comparison with single-agent gemcitabine (Conroy et al., 2010).

1.2 Oxaliplatin mechanism of action

Oxaliplatin is a diamine cyclohexane (DACH) platinum (Pt) derivative that is active in several solid tumor types, especially in some cisplatin-resistant cancers (Machover et al., 1997). It belongs to the category of drugs not cell cycle specific (CCNS), which are able to interact with tumor cells irrespective of the phase of the cell cycle. The mechanism of action of this drug is similar to that of the platinum derivative. It is necessary that it is first converted into its active aqueous form, passing respectively by the intermediate forms mono - and di-chloride. In the active form oxaliplatin is not only able to bind to sulfhydryl groups of proteins and amino, but also and especially to the N atoms of the nitrogen bases of the double helix of DNA, causing the activation of apoptosis (Di Francesco et al., 2002). Its anti-tumor efficacy is mainly attributed to

the formation of adducts DACH-Pt-DNA, which may be formed within the same filament (the majority) or between different filaments (1%). These adducts deform the normal structure of the DNA, and subsequently they block the synthesis and damage repair processes. All this contributes to cell death by apoptosis. Oxaliplatin irreversibly also interacts with other biomolecules such as albumin, cysteine (Cys), methionine (Met) and reduced glutathione (GSH). Then reduced glutathione loses their functionality, promoting the alteration of cellular oxidative equilibrium. Oxaliplatin has a spectrum of action different than the other cancer because its DACH core interacts with proteins in a higher percentage compared to other Pt –derivatives. Lesions oxaliplatin-induced are, therefore, more harmful than those caused by cisplatin or carboplatin (Di Francesco et al., 2002).

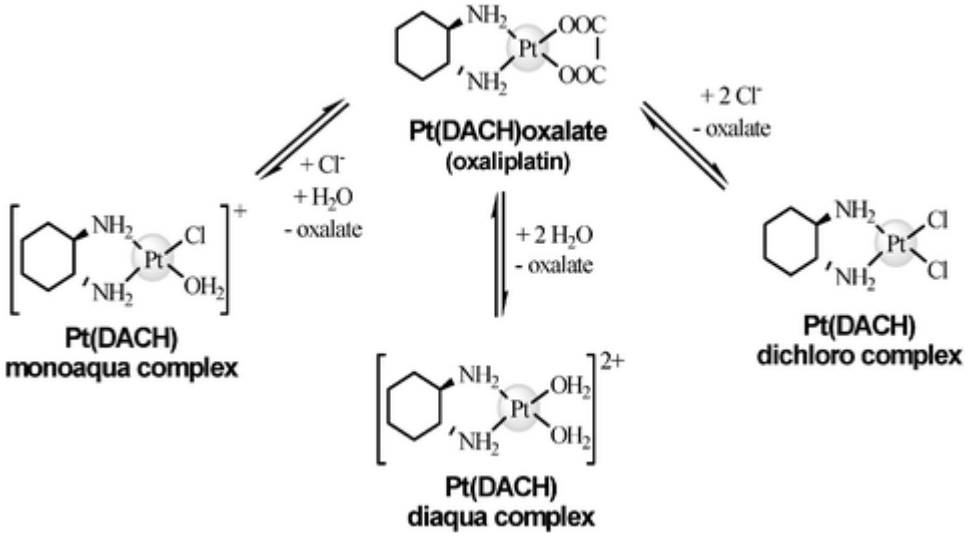


Figure 1. Hydrolisis of oxaliplatin (Esteban-Fernández et al., 2009)

1.3 Oxaliplatin toxicity: dose-limiting neurotoxicity

Oxaliplatin is better tolerated than cisplatin, particularly in terms of renal hematological and gastrointestinal toxicity, but it displays a characteristic pattern of neurotoxicity (Cvitkovic et al., 1999). Oxaliplatin neurotoxicity can manifest as either of two distinct syndromes: a transient, acute syndrome that can appear during or shortly after infusion, and cumulative sensory neuropathy.

About 60–80% of patients develop a stereotyped cold-induced acute neuropathy, that typically resolves within a week (Windebank and Grisold 2008).

Acute oxaliplatin-induced neuropathy has been associated with significant peripheral nerve hyperexcitability, which suggests an immediate pharmacologic rather than structural basis for acute symptoms (Webster et al., 2005). A study that used axonal excitability techniques indicated that neuronal sodium channel dysfunction may play a part in the etiology of oxaliplatin-induced neurotoxicity (Park et al., 2009). A peculiar population of sodium channels (calcium-dependent sodium channels) has been demonstrated to be involved in such neurotoxicity, and the acute action of oxaliplatin is produced by its cytotoxic metabolite oxalate, a hydrolyzable ligand and calcium chelator (Grolleau et al., 2001). Oxalate affects voltage-gated sodium channels and interferes with intracellular divalent cation regulation in neuronal systems and by disrupting their intracellular homeostasis (Gamelin et al., 2002).

Repeated oxaliplatin administrations cause a chronic neuropathy that can affect approximately 50% of patients receiving cumulative doses higher than 1000 mg/m^2 (De Gramont et al., 2000). The chronic administration of oxaliplatin promote the development of a dose-limiting, neuropathic syndrome characterized by paresthesia, dysesthesia, and pain. The sensory neuropathy can lead to functional impairment and even ataxia. The symptoms may be disabling for treated patients, adversely affecting activities of daily living and thereby quality of life (de Gramont et al., 2000, Cassidy and Misset, 2002).

Chronic neuropathy cannot be resolved between cycles (Cersosimo 2005) and sometimes sensory alterations may progress for several months after cessation of treatment ("coasting"; Windebank and Grisold 2008).

The dorsal root ganglia (DRG) are the main target of platinum drug–induced CIPN (chemotherapy-induced peripheral neuropathy; Cavaletti et al., 1992). Although most of the presented results were obtained in cisplatin models, it is likely that the pathophysiology of chronic CIPN induced by carboplatin and oxaliplatin is similar (Cavaletti et al., 1998). The body of the experimental evidence points toward three different putative mechanisms, not necessarily mutually exclusive, because both can eventually produce DRG neuron apoptosis: (i) the formation of platinum intra-strand adducts and inter-strand crosslinks, which influence the tertiary structure of the

nuclear DNA (Ta et al., 2006) altering cell-cycle kinetics (Gill and Windebank,1998), (ii) the interaction with mitochondrial DNA, leading to oxidative stress (Zhan et al., 2007) and, possibly, to p53 increased activity and mitochondrial release of cytochrome-C pathway, independent of Fas receptor activation (McDonald and Windebank, 2002) as well as activation of p38 and ERK1/2 (Scuteri et al., 2009) and (iii) the accumulation of diamine cyclohexane platinum in neurons promotes the alteration of glutathione detoxification system (Gamelin et al., 2007). These mechanisms do not explain the acute, transient symptoms induced by oxaliplatin that have been reported to be secondary to oxalate-induced dysfunction of nodal axonal voltage-gated Na^+ channels (Krishnan et al., 2005). Moreover recent studies indicate that polymorphisms at glyoxylate aminotransferase gene level may be predisposing factors for peripheral sensory neuropathy, but the precise mechanisms responsible for the development of neuropathy in patients receiving oxaliplatin therapy remain unknown (Gamelin et al., 2007).

Principal features of oxaliplatin neurotoxicity

Acute	Chronic
Mild or no axonal degeneration	Axonal neuropathy is similar to other platinum-based chemotherapeutic agents
Effect on sensory neurons and/or motor neurons or muscle cells	Sensory potentials are significantly low whereas motor studies remain essentially normal. At 12-month positive sensory symptoms improve but NCS abnormalities persist
Transient interaction with specific isoforms of ion-channels located in the cellular membrane (i.e. on the dorsal root ganglion neurons) resulting in increase of the Na(+) current	Neurophysiological and neuropathological changes are associated with a significant and dose-dependent reduction in the circulating level of nerve growth factor (NGF), which returned to normal values after neurophysiological and pathological recovery
Block of the maximal amplitude	Striking signs of hyperexcitability are present in motor nerves
Shift of the voltage–response relationship towards more negative membrane potentials	
Increase of peripheral nerve refractory period and consequent hyperexcitability	

Table 1. Principal features of oxaliplatin acute and chronic neurotoxicity

1.4 Prophylaxis and/or treatments

The management of oxaliplatin induced neuropathy is not satisfactory. Drugs used are essentially symptomatic and don't limit significantly the neurodegenerative events, promoting pain perception alterations. Recently Albers et al. (2011), suggested acetylcysteine, Org 2766, and α-tocopherol, as new potential neuroprotective agents, useful for the treatment of platinum drug-induced neuropathy, but they highlighted that data from the clinical trials were insufficient to conclude that any of the neuroprotective agents tested prevent or limit the neurotoxicity of platinum drugs (Albers et al. 2011). Below are listed the pharmacological approaches potentially exploitable.

1.4.1 Ca/Mg infusion

Divalent cations have the ability to modify voltage-gated sodium channels (Gamelin et al 2002). It is hypothesized that the acute neurotoxicity of oxaliplatin is related to the ability of oxalate to chelate calcium. Increases in extracellular calcium have been shown to increase the probability of sodium channel closure decreasing the hyperexcitability of peripheral neurons seen in oxaliplatin-induced neuropathy (Armstrong and Cota 1999).

Magnesium supplementation has been previously studied in preventing cisplatin-induced hypomagnesemia (Lajer and Daugaard 1999). This promising treatment is based on a retrospective study by Gamelin and colleagues (2004) of 161 patients treated with varying regimens of oxaliplatin and 5FU-LV.

Patients had received one of three various oxaliplatin regimens (85 mg/m^2/2 weeks; 100 mg/m^2/2 weeks; or 130 mg/m^2/3 weeks). Ninety-six patients received 1 g each of calcium gluconate and magnesium sulfate intravenously over 15 minutes just before the oxaliplatin infusion. This dose was repeated after completion of the infusion. The percentage of patients with grade 3 distal paresthesias was significantly lower in the Ca/Mg group (7% vs 26%, $p = 0.001$). The acute symptoms of distal and perioral paresthesias were much less frequent. No patients in the Ca/Mg group experienced pseudolaryngospasm. Furthermore, the Ca/Mg group recovered more rapidly from neuropathy especially in the patients receiving 85 mg/m^2 of oxaliplatin (< 2 months).

The Ca/Mg infusions had no bearing on treatment efficacy (Gamelin et al 2004). This was a simple strategy to help ameliorate the symptoms of acute oxaliplatin-induced neuropathy, but further investigation is warranted to determine if this treatment is effective in preventing chronic, cumulative neurotoxicity. It must be borne in mind that this strategy is based on a single retrospective analysis of a nonrandomized study. Prospective, randomized studies (such as the CONCEPT) are underway to validate the benefit of these minerals in ameliorating the neurotoxicity of oxaliplatin.

Saif and Reardon (2004) reported a case of a patient in which oral calcium supplements not only was successful in treating his neurotoxicity, but also the patient was able to receive a cumulative dose of 2500 mg/m^2 (990 mg/m^2 with oral calcium only;).

1.4.2 Glutathione

Glutathione, an important biological antioxidant, is able to prevent the accumulation of platinum adducts in the dorsal root ganglia in rat model (Holmes et al 1998). A single randomized, double-blind, placebo-controlled trial has been done assessing the efficacy of glutathione in the prevention of oxaliplatin-induced neurotoxicity (Cascinu et al 2002). Fifty-two patients were randomized to receive a 1500 mg/m^2 glutathione infusion over 15 minutes or normal saline before oxaliplatin infusion. Oxaliplatin was administered on a bimonthly regimen. The median cumulative dose of oxaliplatin did not differ among the two arms. The glutathione group showed significantly less grade 2 or higher neurotoxicity after 8 cycles of chemotherapy (58% vs 10%). The response rates were similar between the glutathione and placebo groups (26.9% vs 23.1%), suggesting that glutathione does not change the efficacy of oxaliplatin. This finding was of significant importance since glutathione has been shown to affect the efficacy of a variety of antineoplastic interventions (Arrick and Nathan 1984).

1.4.3 Amifostine

Based on the studies of amifostine with cisplatin, its effect has been studied with oxaliplatin. Twenty-one patients with peripheral neuropathies (grade ≥ 2) were treated with amifostine 200 mg/m^2 subcutaneously (SC) over 3 minutes, twice a week for 6 weeks (Penz et al 2001). Patients were continued on amifostine for as long as

improvement was seen. At study entry, 8 patients had grade 3 neurotoxicity and 12 patients had grade 2 neurotoxicity. Among seventeen evaluable patients who completed at least 6 weeks of amifostine therapy, 12 of 17 (71%) patients showed at least minimal (1 grade) improvement in their peripheral neuropathy. One patient reported an increase in peripheral neuropathy from grade 1 to grade 2. Toxicities were manageable with no grade 3 or 4 toxicities observed. Grade 1 or 2 toxicities included: nausea (33%, n = 7); fatigue (9%, n = 2); hypotension (5%, n = 1); and sneezing (5%, n = 1).

1.4.4 Carbamazepine

The theory that oxaliplatin affects voltage-gated sodium channels has led to the use of carbamazepine, a widely used anticonvulsant, to prevent oxaliplatin-induced neuropathy.

Carbamazepine decreases high frequency repetitive firing of action potentials by enhancing sodium-channel inactivation (Macdonald and Kelly 1995). In a small German study, 40 patients refractory to 5-FU were treated with oxaliplatin, 5-FU, and folinic acid as second line therapy (Eckel et al 2003). Ten patients were additionally treated with carbamazepine maintaining serum levels of 3–6 mg/L. The patients in the carbamazepine group were able to receive significantly higher cumulative doses of oxaliplatin (722 mg/m^2 vs 510 mg/m^2; p = 0.02). No neuropathy higher than grade 1 occurred in the carbamazepine group compared with 30% in the control group.

Larger trials need to be conducted to make conclusions about the prophylactic efficacy of carbamazepine in oxaliplatin-induced neuropathy; however, initial data is promising.

1.4.5 Gabapentin

Gabapentin, another widely used anticonvulsant, appears to affect the release of gamma-aminobutyric acid (GABA). The side effect profile and therapeutic index of gabapentin make it more tolerable and easier to administer than carbamazepine, thus making it more attractive to use for prophylaxis of oxaliplatin-induced neurotoxicity. In a pilot study, 15 patients were treated with oxaliplatin (85 mg/m^2/devery 2 weeks) plus 5-FU, and folinic acid as second therapy for advanced colorectal cancer (Mariani

et al 2000). Gabapentin at a dose of 200 mg/d was started at the onset of neuropathic symptoms. If the patients' symptoms did not resolve in a period of 3 days, then the dose was increased to 300 mg/d. All patients treated with gabapentin had resolution of their symptoms, and no patients had to stop therapy secondary to neurotoxicity. In another study presented at ASCO (2005), 115 patients with chemotherapy-induced peripheral neuropathy were randomized in a double-blind, placebo-controlled trial to either: gabapentin (target dose = 900 mg three times a day [TID]) for 6 weeksthen crossover to placebo for 6 weeks (n = 57) or treatment in the reverse order (n = 58) (Wong et al 2005). A 2-week washout occurred between crossover treatments. The coprimary endpoints were the average daily pain numerical analogue intensity rating (0 = no pain to 10 = worst pain imaginable) and the ECOG toxicity rating for sensory neuropathy (0 = none to 3 = severe). The results of the study showed that gabapentin did not significantly improve the co-primary endpoints of pain intensity (–0.5 vs –1.0 change from baseline to week 6 for patients on gabapentin and placebo respectively, p = 0.18) or the ECOG toxicity rating for sensory neuropathy (–0.2 vs –0.1 for gabapentin and placebo respectively, p = 0.38). Patients on gabapentin reported significantly more nystagmus (p = 0.009) and dizziness (p = 0.02). Therefore, the study was not able to confirm the benefit of the use of gabapentin in ameliorating peripheral neuropathy.

1.4.6 Other neuromodulatory agents

Other agents including acetyl-L-carnitine (ALCAR), and α-lipoic acid have shown some promise in small trials. Maestri et al (2002) presented at ASCO (2002) a study evaluating the role of ALCAR in the management of neurotoxicity associated with oxaliplatin. They found that ALCAR was effective in treating patients with established chemotherapy-induced peripheral neuropathy (Maestri et al 2002). Twenty patients were followed with neurotoxicity defined by the WHO criteria. The patients had been treated with various agents including platinum compounds, taxanes, and vinca alkaloids. All were treated with ALCAR 1 g infusion over 1–2 hours for at least 10 days. Sixteen of 20 patients showed at least one grade improvement in their peripheral neuropathy.

Similarly α-lipoic acid has shown beneficial effects in patients with established platinum-induced chemotherapy (Gedlicka et al 2002). In a study of 15 patients, neurologic symptoms improved (by at least one grade) in seven patients with grade 2 peripheral neuropathy and in one patient who suffered from grade 3 symptoms. The median time to response was 4 weeks (range, 3–12 weeks), and the median duration of treatment with α-lipoic acid was 2 months (range, 1–4 months).

1.4.7 Education of the patients

Education of the patients and care givers, including both physician and nursing staff, about symptoms resulting from oxaliplatin-induced neurotoxicity is fundamental. It is equally essential that they be familiar with measures to be taken to manage these events. Patients must be instructed to avoid exposure to cold objects, environment, and liquids.

Reassurance that the acute symptoms of neurotoxicity are transient is very important. The profession of the patient, such as a meat handler, should be considered before offering such an agent. Additionally, patients should be routinely questioned on the events or presence of subjective symptoms. Such questions must be focused on the nature (paresthesias, dysesthesias, hyperesthesias, pain, numbness, muscle contractions, and weakness), location (extremities, perioral area), relationship to cold (if any), time course (onset in relation to oxaliplatin infusion, duration, transient versus persistent), and severity (presence of functional impairment). In case of reported "difficulty breathing" or "laryngospasm", it is important to distinguish between cold-related symptoms (pharyngolaryngeal dysesthesia, muscular contractions) and noncold-related symptoms (muscular contractions). In the latter case, if a cutaneous rash is present, a differential diagnosis of acute hypersensitivity associated with oxaliplatin is mandatory. Checking O_2 saturation may be useful in ruling out the presence of an allergic reaction associated with Quincke's edema. Furthermore, this would substantially help reassure the patient (Saif and Reardon 2005).

1.5 The role of glia in the development and maintenance of neuropathic pain

Neuropathic pain refers to a variety of chronic pain conditions. It can originate from neuronal tissue damage or a dysfunction in the nervous system (Hansson et al., 2001) The abnormal perception of neuropathic pain is characterized as being allodynic (a typically nonpainful stimulus is perceived as painful), hyperalgesic (a normally painful stimulus is exaggerated) or as spontaneous (shock-like, stabbing or burning pain sensations that are unrelated to a known stimulus; Cavenagh et al., 2006). The sensation of the neuropathic pain may or may not be localized to the dermatomal distribution of the affected nerve(s). Studies indicate that a communication exists between the immune system and the nervous system (Safieh-Garabedian et al., 2002).

Although multiple conditions may generate neuropathic pain, a common underlying mechanism is the presence of inflammation at the site of the damaged or affected nerve(s). This inflammatory response initiates a cascade of events resulting in increased local perfusion, increased capillary permeability, and concentration and activation of innate immune cells at the site of tissue injury, irritation, or infection. Immunoactive substances, such as cytokines, neurotrophic factors, and chemokines, released at the site of injury have local actions and can initiate a systemic immune response.

The resultant neuroinflammatory environment can cause activation of microglia and astrocytes, glial cells located in the spinal cord and brain, which appear to play a prominent role in nociception (Vallejo et al., 2010).

Neurons and glial cells are two cell types in the nervous system that have close interactions on a cellular and molecular level. Neurons are cells specialized to conduct electrochemical impulses. Glial cells, also known as neuroglia, are non-conducting cells that were initially only known to provide support; however, recent evidence has shown that glial cells also provide nutrition, protection, and insulation to the neurons of the central nervous system (CNS; Vallejo et al., 2010). Glial cells constitute 70% of the total cell population in the brain and the spinal cord (DeLeo and Colburn, 1998). Glial cells can be subdivided into two primary categories: microglia, comprising 5% to 10% of the glial population, and macroglia, which include astrocytes and

oligodendrocytes (Moalem and Tracey, 2006). Furthermore, astrocytes and microglia are known to play a role in the development, spread, and potentiation of neuropathic pain (Watkins et al., 2001; McMahon et al., 2005).

When myeloid progenitor cells migrate to the peripheral nervous system (PNS), they may differentiate into dendritic cells or macrophages. However, when the same bone marrow-derived progenitor cells travel to the CNS, they differentiate into microglia which act similarly to macrophages when they are activated (Nakajima and Kohsaka 2001).

Under normal homeostatic conditions, microglia are in a resting, sessile state and have small soma with fine or thin-branched processes. Microglial cells migrate to the central terminals of afferent peripheral nerves responding to pain signals and undergo activation. Upon activation microglia undergo a number of morphological and functional changes facilitating isolation of injured cells and eliminating potential pathogens (Davalos et al., 2005).

These changes include mobilization and proliferation and induce phagocytic ability of microglia (Kreutzberg, 1996). At the site of injury, activated microglia can project processes, through an adenosine 5′-triphosphate (ATP)-mediated elongation, in order to isolate the injured cells (Davalos et al., 2005). Microglia play a pivotal role in the trauma-induced pain models, where the inflammatory component is preeminent and microglia acts as a promoter in the initial phase of pain facilitation (Pacini et al., 2010). In a rat model of oxaliplatin-induced neuropathy (Cavaletti et al., 2001), where the inflammatory component is slight, microglia and astrocytes seem to work hand-in-hand, at least at the initial phase of spinal cord sensitization, but although microglia conclude the task within the 14th day of oxaliplatin administration, astrocytes are still active and will likely contribute to the maintenance of persistent pain states (Di Cesare Mannelli et al., 2013b).

After peripheral nerve injury, proliferation of activated microglia was found on the ipsilateral dorsal horn (DH), while the contralateral DH and naive animals displayed weak microglial activation (Beggs and Salter 2007). Activated microglia also display a change in surface markers, membrane bound or embedded proteins, compared with resting stage microglia. Activated surface markers can include complement receptor 3 (also known as CD11b/ CD18, Mac-1, ITGAM or integrin alpha-M; Coyle 1998)

which is involved in phagocytosis (Wieseler-Frank et al., 2005), toll-like receptor 4, which is involved in pathogen recognition, CD14, also involved in pathogen recognition (Raghavendra et al., 2004), CD44 involved with adhesion and migration (Sweitzer et al., 2002), and up-regulation of MHC I and II (Sweitzer et al., 2002) which are involved in antigen presentation to T cells (Coull et al., 2005). These better prepare the glial cells to eliminate invading microbes and to aid in phagocytosis (Wieseler-Frank et al., 2005). The activation of microglia additionally triggers the secretion of a variety of signaling peptides such as cytokines, neurotrophic factors, and chemokines. The production and subsequent release of proinflammatory cytokines like interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) from activated microglia cells lead to the activation of neighboring astrocytes and influenced neuronal functionality (Watkins et al., 2003).

Astrocytes are the majority of glial cells in the CNS; however, the development and function of astrocytes remains largely uncharacterized. Astrocytes are phagocytic cells that play an important role in neuronal development as well as in establishing and maintaining the blood brain barrier (BBB; Cahoy et al., 2008) In a resting state, astrocytes isolate neurons and oligodendrocytes to help maintain the microenvironment of the CNS by regulating extracellular ion concentrations of K^+ and Ca^{2+} as well as neurotransmitter concentrations via uptake. In a basal state, astrocytes have thin processes. Upon activation, these cells undergo hypertrophy, proliferate, and increase expression of intermediate filaments such as the glial fibrillary acidic protein, an astrocyte-specific activation marker (Garrison et al., 1994). These functions provide important links to antigen presentation to T cells and may aid T cell crossing the BBB.

The activation of astrocytes results in the prolongation of a pain state. Resting astrocytes express basal levels of cytokine receptors. IL-1β and possibly interleukin-18 (IL-18), released from activated microglial cells, bind to interleukin-1 (IL-1) receptors located on the astrocyte membrane, inducing a series of intracellular events culminating in the activation of the astrocyte (Miyoshi et al., 2008). TLR, expressed in microglial cells, may trigger the synthesis of IL-18, a member of the IL-1 family, via the activation of p38 mitogen activated protein kinase (p38MAPK), known to induce expression of proinflammatory cytokines such as IL-1β and IL-6. Miyoshi et al.(2008) reported that intrathecal injection of IL-18 induces tactile allodynia and astrocyte

activation. These intracellular events result in the secretion of IL-1β, IL-6, and TNF-α by astrocytes, as well as the expression of inducible nitric oxide (NO) synthetase, to further propagate the inflammatory response and prolong the pain state.

Another unique characteristic of astrocytes is their role in both the deactivation of glutaminergic activity by the uptake of extracellular glutamate and the synthesis of glutamate from glucose. Glutamate (the main excitatory neurotransmitter in the brain and the spinal cord) content increases in the DH during chronic pain. Pyruvate carboxylase, an enzyme involved in the synthesis of glutamate, is expressed in astrocytes but not in neuronal cells (Hertz and Hansson. 2007). Glutamate activates several ionotropic and metabotropic membrane receptors. Of particular interest is the ionotropic N-methyl-D-aspartate receptor because of its crucial role in central sensitization of spinal cord nociceptive neurons as well as activation of astrocytes via an influx of Ca^{2+} into the cells (Petrenko et al., 2003). An influx in Ca^{2+} has an important role in pain signaling by promoting neurotransmitter release and modulating cell membrane excitability.

1.5.1 Activation of glia cells after nervous tissue injury

The activation of microglia and astrocytes can occur following physiological changes in the body, such as trauma in the CNS, ischemia, inflammation, and infection. The activation of these glial cells is most often implicated in the development, spread, and potentiation of neuropathic pain (Watkins et al., 2001). Microglia and astrocytes are generally activated in the DH after a peripheral nerve injury occurs. After receiving a pain stimulus, peripheral neurons transmit “pain” signals to the DH of the spinal cord, releasing neurotransmitters such as calcitonin gene-related protein (CGRP), substance P, glutamate, gamma amino butyric acid, serotonin (released from descending pain pathways), and ATP. These neurotransmitters initiate the activation of glial cells in the area of the synapse, further sensitizing postsynaptic neurons. Various mechanisms by which glial cells are activated have been suggested. These include:

1) Chemical mediators including substance P, CGRP, NO, purinergic agents (such as ATP), glutamate, and endogenous opioid peptides released at the time of injury travel through or between afferent neurons, not only affecting synaptic transmission but also activating glial cells (Gou et al., 2007).

2) Glial activation can occur via shifts in intracellular and extracellular ion concentrations. An increase in afferent neuronal input causing an elevation of extracellular K^+ leads to increased K^+ uptake by astrocytes, resulting in membrane depolarization, morphologic changes, and possibly activation (Hansson 2006) Furthermore, K^+ has been shown to induce microglial activation in rat hippocampal tissue *in vitro* (Abraham et al., 2001). Similarly, an influx of Ca^{2+} results in the activation of both astrocytes and microglia, with concomitant changes in morphology and cellular function. Pro-inflammatory agents generated and released by activated glial cells can further activate nearby glial cells.

3) Previous studies have shown that peripheral injury results in astrocyte activation in the trigeminal complex of the brain stem (Cao et al., 2008). Interestingly, proximal blockade of primary afferent input following a peripheral nerve injury fails to inhibit glial activation at both the spinal and supraspinal levels. These studies suggest that supraspinal-activated astrocyte cells may potentially modulate neuropathic pain by further activation of glial cells in the spinal cord via descending pathways (Wei et al., 2007)

4) It has been shown that increased permeability of the BBB after an injury allows peripheral macrophages to migrate, proliferate, and differentiate into activated glial cells in the brain (Cao et al., 2008). In addition, peripherally generated inflammatory agents, outside of the neuronal afferent pathway, can activate glial cells in the CNS. For example, a proximal anesthetic block fails to inhibit either spinal cyclooxygenase gene expression or prostaglandin E2 release into the cerebrospinal fluid.

Interestingly, acute pain, such as a paper cut or a needle prick, will not activate glial cells (Holguin et al., 2004). However, following a more serious injury, glial cells exhibit dynamic plasticity and switch from a resting state to become active in the modulation of neuronal activity (Ren and Dubner 2008). Once activated, glial cells change their morphology, via hypertrophy and potentially retraction of the processes, and synthesize specific cell markers and kinases, some having an active role in initiating and potentiating an immune response.

Both microglia and astrocytes are involved in neuropathic pain pathways. After a threshold stimulus, activated glial cells release inflammatory stimulants such as cytokines, prostaglandins, neurotrophic factors, ATP, NO, d-serine, and glutamate

(Werry et al., 2006; Bhangoo et al., 2007; Ren and Dubner 2008). These inflammatory stimulants play a critical role in the development and maintenance of central sensitization and hyperalgesia (Holguin et al., 2004) by altering the polarization characteristics of the afferent neurons and thus modulate the transmission of painful stimuli to the CNS (Perea and Araque 2007). For example, astrocyte activation leads to increasing intracellular Ca^{2+} which stimulate a calcium-dependent glutamate release, resulting in an inward current produced in adjacent neurons (Parpura and Haydon 2000).

Current research suggests that microglia are involved in the early development, whereas astrocytes sustain the chronicization of neuropathic pain (Ledeboer et al., 2005). Microglial activation leads to the release of signaling proteins, such as IL-1β, into the cell interstitium and to some extent the CSF. These signaling proteins bind to specific sites on the astrocyte membrane initiating cell activation (Sama et al., 2008) Upon activation, a positive feedback cycle occurs whereby astrocytes release inflammatory mediators, e.g., TNF-α, which in turn can activate other glial cells (Sama et al., 2008) Astrocyte activation is accompanied by a decrease in microglial activity over time (Tanga et al., 2004). In an animal model, intrathecal administration of activated microglial cells decreased pain threshold while a similar application of activated astrocytes did not (Narita et al., 2006), further demonstrating that activated astrocytes are not predominantly involved in the development of a pathological pain state, but rather potentiation of a pain state.

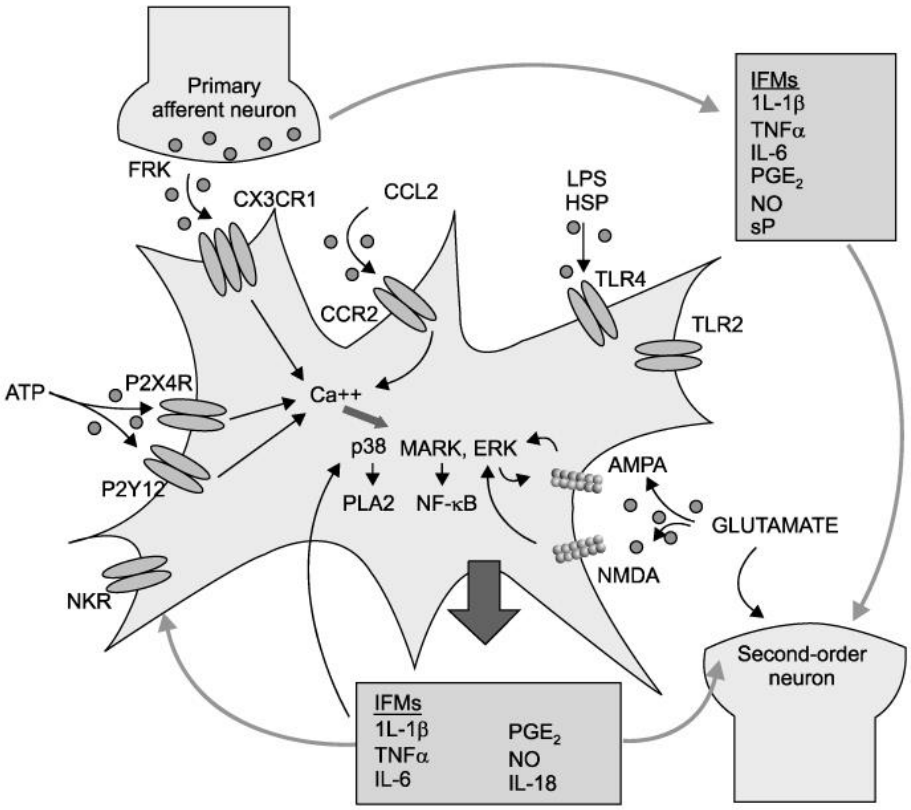


Figure 1. Activation and response of microglia in neuropathic pain. Nerve injury upregulates multiple receptors, including the chemokine receptors CX3CR1, P2X/Y, CCR2 and TRL4 in spinal microglia, and it induces the release of frackalkine and neurotransmitters from the primary afferent neuron that also activate microglia. Activation causes microglial cell increase intracellular calcium, and initiates the p38 MAPK/ERK pathway, which is necessary for the release of pre-inflammatory substances. The activated microglia release several pro-inflammatory cytokines, chemokines and other agents that modulate neural transmission by affecting presynaptic release of neurotransmitters and/or postsynaptic excitability. (Daehyun et al., 2009).

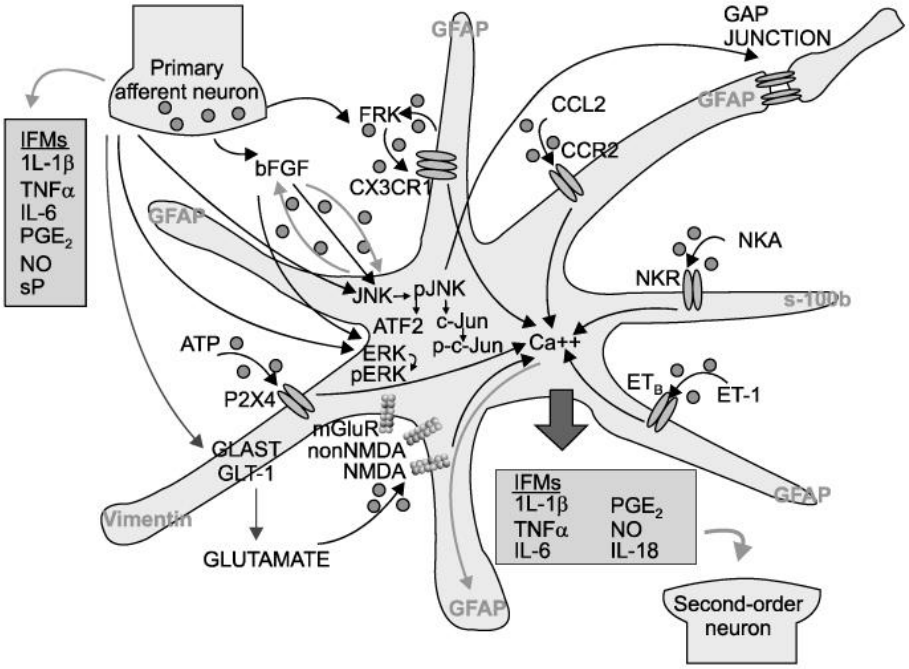


Figure 2**.** Activation and response of astrocytes in neuropathic pain. Nerve injury upregulates several receptors, such as the chemokine receptors CX3CR1, P2X4 and CCR2, ETB, NKR, and NMDA in spinal astrocytes, and it increases markers such as GFAP, vimentin and S-100β. Also, nerve injury initiates a persistent downregulation of the glutamate transporters, such as GLT-1 and GLAST. Astrocytes respond to ongoing synaptic activity by mobilizing internal Ca^{++}, and this in turn leads the release of glutamate, ATP, TNF-α, IL-1β, IL-6, NO, and $PGE_{2.}$ (Daehyun et al., 2009).

1.6 Role of the oxidative stress in platinum drug neurotoxicity

Oxidative stress is generally described as an imbalance between the production and the elimination of free radicals, such as reactive oxygen species (ROS) and nitrogen species (RNS). Cellular metabolism is the main source of free radicals and the endogenous antioxidant defences maintain the physiological redox equilibrium. When this oxidative state is lost, there is the generation of oxidative stress, able to alter the intracellular molecules functionality, including DNA, RNA, lipids and proteins (Veskoukis et al., 2012)

Among the free radicals, ROS are the most abundant and include the superoxide anion ($O^{2-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), singlet oxygen ($O^{2\cdot}$) and the ozone (O_3) (Lizama-Manibusan and McLaughlin 2013).

$O_2 + 1e^- \rightarrow O_2^-$ (superoxide anion)

$O_2^- + 2e^- + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide)

$H_2O_2 + O_2^- \rightarrow 2OH^{\cdot} + O_2$ (hydroxyl radical)

$NO + O_2^- \rightarrow O\text{-}ON\text{-}O^-$ (peroxynitrite anion)

Generation of free radicals.

Oxidative equilibrium unbalance is crucial for the pathogenesis of neuropathies. ROS can induce major alterations in the mitochondria functionality, as well as induce the synthesis and release of proapoptotic factors such as kinase-1, c-jun NH2-kinase and p38 (Benhar et al., 2001; Tobium et al., 2001), bringing activation of the p53 pathway and/or the mitochondrial apoptotic cascade (Jabs et al., 1999). A mitochondrial damage leads to further excessive generation of ROS: the blockade of the respiratory chain promotes the formation of superoxide anion and further mitochondrial damage. The increased exposure to ROS, the deficit of energy production and altered membrane permeability are responsible for initiation of apoptosis with mitochondrial release of pro-apoptotic factors such as cytochrome C and other (Jabs et al., 1999). In one study it was evaluated the hypothesis that Drp-1 (dynamin -related protein 1), a

GTPase that catalyzes the process of mitochondrial fission, which is an important mechanism for the production of reactive oxygen species (ROS), may play a central role in the development of neuropathic pain symptoms (Kim et al., 2004; Yowtak et al., 2011). Ferrari and colleagues have demonstrated a marked reduction in mechanical hyperalgesia induced by oxaliplatin (2 $mgkg^{-1}$ intravenous) of the rat, by intrathecal injections of mdivi-1, highly selective inhibitor of Drp-1. Furthermore, to evaluate the correlation between the mechanical hyperalgesia induced by oxidative stress and the role of the enzyme Drp-1, the rats were injected intradermally with hydrogen peroxide, powerful generator of ROS in the cells and potent inducer of mechanical hyperalgesia. Even in this case intrathecal injections of mdivi-1 have reduced markedly hyperalgesia (Ferrari et al., 2011).

In other animal models of neuropathy from oxaliplatin was identified mitochondrial dysfunction in axons of peripheral nerves, described as swelling and increased mitochondrial vacuoles. Moreover the sciatic nerve of the rat, after 3-4 weeks of treatment with oxaliplatin, showed a decreased activity of complex I and II of mitochondrial respiratory chain, as well as deficit of ATP production. In these studies also prophylactic treatment with an antioxidant Acetyl-Carnitine, which has been shown to reduce the development of neuropathy induced by oxaliplatin, is preventing mitochondrial damage (Zheng et al., 2011).

Some studies have focused on the role of antioxidants in the mechanism of cisplatin toxicities. Recent evidence indicates that cisplatin-induced side effects are, at least in part, the result of the formation of oxygen free-radicals (Smoorenburg et al., 1999). In animals, supplementation with antioxidants, such as vitamin E (α-tocopherol), vitamin C, and selenium, seems to protect against cisplatin-induced renal and ototoxicity (Rybak et al., 2000). Moreover, data obtained in human studies indicates that cisplatin treatment induces a fall in plasma antioxidant levels because of oxidative stress (Weijl et al., 1998). It is interesting to note that the clinical and neuropathologic features observed in cisplatin-induced neuropathy are similar to those observed in vitamin E deficiency–induced neuropathy. In humans, vitamin E deficiency syndromes (eg, lipid malabsorption, cholestatic liver disease, abetalipoproteinemia, short bowel syndrome, cystic fibrosis, and familial sporadic vitamin E deficiency) are characterized by a peripheral sensory neuropathy with ataxia, paresthesia in a stocking and glove

distribution (paresthesias and numbness in feet and hands), and loss of reflexes caused either by the retrograde degeneration of the large caliber axons in peripheral nerves or by degeneration of the posterior columns of the spinal cord (Traber et al., 1987). Pathologic studies indicate that the dorsal-root ganglia neurons are the primary target of vitamin E deficiency (Muller et al., 1983).

Pace et al. (2002) described that vitamin E supplementation significantly protects against cisplatin-induced peripheral neurotoxicity and reduces the incidence and intensity of neuropathic signs and symptoms. Nonetheless, the author suggested that the efficacy of neuroprotection with vitamin E supplementation has to be assessed in larger studies.

Recently Albers et al. underlined, recently, that clinical data are insufficient to conclude that any of the purported neuroprotective agents for the prevention of the platin drugs- neurotoxicity (including antioxidant compounds like acetylcysteine, glutathione and Vitamin (E) are effective (Albers et al., 2011).

2.Aim of study

Oxaliplatin is a platin-organic drug with anti-neoplastic properties used for colo-rectal cancer (Machover et al., 1996). In respect to other anti-neoplastic platinum derivates, oxaliplatin has only a mild hematological and gastro-intestinal side-effects. Its limiting side effects are the neurotoxicity and the subsequent nerve hyperexcitability whom result in a neuropathic syndrome (Gamelin et al., 2002). In particular, oxaliplatin induces an acute neurologic symptom complex, that disappears spontaneously. Moreover repeated oxaliplatin administrations cause a chronic neuropathy that can affect approximately 50% of the patients receiving cumulative doses higher than 1000 mg/m^2 (Cascinou et al., 1995; Beg et al., 2008). Chronic treated patients manifest a characteristic set of symptoms, such as paresthesia, dysesthesia, and pain, that affect significantly their quality of life. Only the interruption of the life-saving therapy limits the entity of the symptomatology (Cersosimo 2005). Randomized trials demonstrating that prophylactic or therapeutic effect of antihyperalgesic drugs on oxaliplatin's cumulative neurotoxicity are still lacking or inconclusive (Albers et al., 2011). An important limit in this field is the insufficient information on the molecular basis of the neuropathy. In particular there isn't a clear pathological target exploitable for the antineuropathic therapy (Albers et al., 2011). The oxidative hypothesis, as biomolecular alteration at the base of oxaliplatin-induced neuropathy, is still a matter of debate (Albers et al., 2011). Antioxidant compounds, like N-acetylcysteine, and vitamin E (α-tocopherol) have been tested as possible therapeutic approaches (Cascinu et al., 1995; Cascinu et al., 2002; Argyriou et al., 2006; Kottschade et al 2011). However, the major bias of the clinical trials resides in their small size and/or in their short term follow-up. Direct data about oxidative damage induced in vivo by platin derivatives are however still lacking (Albers et al., 2011).

The aim of our study is to verify whether oxidative stress could be responsible for the pain perception alterations. For this purpose in a rat model of oxaliplatin-induced neuropathy (Cavaletti et al., 2001), firstly, we will characterize the neuropathic syndrome, evaluating the response to a noxius and non-noxius stimulus and the motor coordination. Moreover we will measure the oxidative damage induced by oxaliplatin at plasmatic and nervous levels, considering the principal biological components, such as proteins, lipids and genome. Aimed to highlight the relationship between oxidative stress and pain perception dysregulation oxaliplatin-induced we will use, as

therapeutic agent, two well known natural antioxidants, α-tocopherol and silibinin. Silibinin is the principal component of the silymarin complex, an active extract from the seeds of the plant milk thistle (Silybum marianum).

Aimed to deep inside the biomolecular alterations at the base of oxaliplatin-induced neurotoxicity and to screen new active compounds, we will try to set up a nervous cellular model of oxaliplatin neurotoxicity. In specific brain areas of neuropathic rats astrocyte cells resulted activated after repeated treatment with oxaliplatin (Di Cesare Mannelli et al., 2013). It is well known that glia have a pivotal role in pain chronicization and astrocytes may be involved in the maintenance of neuropathic pain (Marchand et al., 2005; Scholz et al., 2007). For that purpose we will chose to investigate oxaliplatin neurotoxicity on isolated primary cultures of cortical astrocytes and using the neuronal-derived cell line SH-SY5Y. In these cell cultures firstly we will evaluate the cell viability and the oxidative stress after oxaliplatin incubation. For the oxidative stress we will considerer the measurement of superoxide anion, free radical generated at mithocondrial level, and the oxidative damage at protein, lipid and genome level, similarly to the *ex-vivo* analysis. Aimed to investigate the apoptotic activation promoted by the platinum drug in nervous cells, we will measure the activity of caspase-3, end effector of the apoptotic processes, after the incubation with the chemotherapic agent. Then we will exploit the antioxidants properties of α-tocopherol and silibinin, to prevent the hypothetical oxidative stress and the cytotoxicity, in terms of caspase-3 activation, promoted by oxaliplatin.

Aimed to investigate if silibinin or α-tocopherol were able to alter the oxaliplatin efficacy we will used a human adenocarcinoma colo-rectal cell line (HT-29 cells), the biological target of the platinum drugs. For that purpose we will treat tumoral cell with oxaliplatin in presence of the antioxidants compounds and we will measure the cell viability and the activation of caspase-3. Moreover, both the intrinsic and extrinsic apoptotic pathways will be studied in HT-29 and astrocytes to highlight possible different mechanisms of programmed death in tumoral vs normal cells.

3.Material and methods

3.1 Animals

For all the experiments described below, male Sprague-Dawley rats (Harlan, Varese, Italy) weighing approximately 200 to 250 g at the beginning of the experimental procedure were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Four rats were housed per cage (size 26 x 41 cm); animals were fed with standard laboratory diet and tap water ad libitum, and kept at 23 ± 1°C with a 12 hour light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278- 01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Animals were anesthetized before cervical dislocation. All efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2 Paw pressure test

The nociceptive threshold in the rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al (1988). Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe by a mechanical device. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while rats were lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in grams. Rats scoring below 40 g or over 75 g during the test before drug administration were rejected (20%). For analgesia measures, mechanical pressure application was stopped at 120 g. Experiments were performed blind.

3.3 Cold Plate Test

The animals were placed on a stainless box (12- x 20- x 10- cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4 ± 1 °C. Pain-related

behaviors (i.e. lifting and licking of the hind paw) were observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 seconds.

3.4 Rota-Rod Test

Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 rpm. The integrity of motor coordination was assessed on the basis of walking time and the number of falls from the rod for a maximum of 10 minutes (600 seconds). After a maximum of 6 falls from the rod, test was suspended and the time was recorded.

3.5 Oxaliplatin Model

Rats were treated with 2.4 mg kg^{-1} oxaliplatin, administered intraperitoneally (i.p.) for 5 consecutive days every week for 3 weeks (15 i.p. injections; Cavaletti et al., 2001). Oxaliplatin was dissolved in 5% glucose solution. Control animals received an equivalent volume of 5% glucose i.p. Behavioral and biochemical tests were performed at the 21^{st} day. The time course of behavioral measures revealed that pain began on day 14 but not uniformly in all animals.

3.6 Drug Treatments for *in vivo* experiments

Silibinin (Sigma-Aldrich, Milan, Italy) and α-tocopherol (Sigma-Aldrich, Milan, Italy) were used at 100 mg kg^{-1}. Both drugs were suspended in 1% carboxymethylcellulose sodium salt (CMC) and administered by the per os (p.o.) route. Repeated treatment (chronic) consisted of a daily administration following the same protocol described for oxaliplatin from the first up to the 20th day. Behavioral and biochemical tests were performed 24 hours after the end of treatments. Acute treatment consisted in a single p.o. administration of silibinin and α-tocopherol (100 mg kg^{-1}) on day 21 of oxaliplatin injection. Control animals received an equivalent volume of 1% CMC p.o.

3.7 Tissue Processing

After behavioral tests, animals were sacrificed, blood was collected in heparin-treated tubes, and plasma fraction was isolated by centrifugation. The sciatic nerve and the lumbar portion of spinal cord were isolated, immediately frosted in liquid nitrogen, and fragmented. Part of the obtained powder was treated with TRI-Reagent (Sigma-Aldrich, Milan, Italy) and processed for DNA extraction.

The remaining part was homogenized in lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, Complete Protease Inhibitor (Roche), and the homogenate was incubated on ice for 30 minutes. Then, the suspension was sonicated on ice using 3, 10-second bursts at high intensity with a 10-second cooling period between each burst. The obtained material was used for lipid peroxidation assay and, after centrifugation (13000 g for 15 minutes at 4 °C), for Western blot analysis

3.8 Cell cultures

The human neuroblastoma cell line SH-SY5Y and human colon cancer cell line HT-29 were obtained from the American Type Culture Collection (Rockville, MD, USA). SH-SY5Y were cultured in Dulbecco's modified Eagle's medium (DMEM)/HAM F12 supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Italy).

HT-29 were cultured in high-glucose DMEM with 10% FBS in a 5% CO_2 atmosphere at 37 °C. Media contained 2 mM L-glutamine, 1% essential amino acid mix, 100 $IUml^{-1}$ penicillin, and 100 $\mu g \cdot ml^{-1}$ streptomycin (Sigma, Germany).

Primary cultures of astrocytes were obtained according to the method described by McCarthy and de Vellis (McCarthy and de Vellis 1980). Briefly, the cerebral cortex of newborn (P1–P3) Sprague–Dawley rats (Harlan, Italy) was dissociated in Hanks' balanced salt solution containing 0.5% trypsin/EDTA and 1% DNase (Sigma) for 30 min at 37 °C. The suspension was mechanically homogenized and filtered. Cells were plated in high-glucose DMEM with 10% FBS. Confluent primary glial cultures were used to isolate astrocytes, removing microglia and oligodendrocytes by shaking. The purity of astrocyte cultures was determined immunocytochemically by staining for GFAP (Dako, Denmark). Cells were fixed in 4% paraformaldehyde, then incubated with the antibody (1:500), and visualized using Alexa Fluor-conjugated secondary

antibody. Nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride. GFAP-positive cells were 90% in astrocyte cultures. Experiments were performed 21 days after cell isolation. Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. The ethics policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence Assurance No. A5278-01).

Cells were incubated with 0.1–100 μM oxaliplatin as described for each measure (see below) in the absence or presence of 10 μM silibinin or α-tocopherol (Sigma). Time intervals and concentrations used in each set of experiments were chosen with respect to the method sensibility and specificity.

3.9 Lipid Peroxidation (Thiobarbituric Acid Reactive Substances, TBARS)

Thiobarbituric acid reactive substances (TBARS) were quantified, as an index of lipid peroxidation, in plasma, tissue homogenates and cells (SH-SY5Y and astrocytes) as described by Pan et al., (Pan et al., 2009) with some minor modifications.

100 ml of plasma or 100 mg of tissue homogenate were added to 4 mL reaction mixture consisting of 36 mM thiobarbituric acid (Sigma-Aldrich, Milan, Italy) solubilized in 10% CH_3COOH, 0.2 % SDS, pH was adjusted to 4.0 with NaOH. The mixture was heated for 60 minutes at 100 °C and the reaction was stopped by placing the vials in ice bath for 10 minutes. After centrifugation (at 1600 g at 4 °C for 10 minutes) the absorbance of the supernatant was measured at 532 nm (Perkin-Elmer spectrometer, Monza, Italy) and TBARS were quantified in mmoles/milligram of total protein using 1,1,3,3- tetramethoxypropane as standard.

SH-SY5Y cells or astrocytes were plated in 25-cm^2 cell culture flasks (10^6 cells/flask) and grown until confluent. Cells were incubated with 10 μM oxaliplatin in presence or absence of 10 μM silibinin or α-tocopherol for 16 hours. After incubation, the cells were scraped and the cell suspension underwent a freeze/thaw cycle. The suspension was added to 4 ml reaction mixture, and processed in the same condition described above. Protein concentration of each sample (plasma, tissue or cells) was measured by bicinchoninc acid (BCA; Sigma-Aldrich, Milan, Italy) assay.

3.10 Carbonylated Protein Evaluation

Plasma or nervous tissue proteins extract were quantified by BCA. Twenty μg of each sample were denatured by 6% SDS and derivatized by 15-minute incubation with 2-4 dinitrophenyl hydrazine (DNPH; Sigma-Aldrich, Italy) at room temperature. Samples were separated on a 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis and transferred onto nitrocellulose membranes (Biorad, Italy). Membranes were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) and then probed overnight with primary antibody specific versus DNPH (Sigma-Aldrich, Italy) 1:5000 in PBST 5% nonfat dry milk. After washing with PBST, the membranes were incubated for 1 hour in PBST containing the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000; Cell Signalling, USA) and again washed. ECL (Pierce, USA) was used to visualize the peroxidase-coated bands. Densitometric analysis was performed using the ‘‘Scion Image’’ analysis software.

For each experiment the density of all bands showed in a lane was reported as mean. For plasma samples Ponceau-stained membranes were used as loading control. β-actin normalization was performed for nervous tissue samples.

SH-SY5Y cells or astrocytes were plated in six-well plates ($5 \cdot 10^5$ cells/well) and grown until confluent. Carbonylated proteins were evaluated after 16 h incubation with 10 μM oxaliplatin in presence or absence of 10 μM silibinin or α-tocopherol. Protein concentration was measured by BCA assay. Twenty micrograms of proteins of each sample was denatured by 6% SDS and derivatized by 15 min incubation with 2,4-dinitrophenyl hydrazine (DNPH; Sigma–Aldrich) at room temperature. Samples were processed similarly to the tissue homogenate sample and β-actin was used as loading control.

3.11 DNA Oxidation

One hundred μg DNA of each tissue homogenate sample were digested for 2 hours (37 °C) in 25 mM CH_3COONa buffer, pH 4.8, containing 1 mM $ZnCl_2$ and 1.1 units of nuclease P1 (Sigma-Aldrich, Milan, Italy). pH was then adjusted to 8.0 using Tris 1.5 M. One unit of alkaline phosphatase (Sigma-Aldrich, Italy) was added and incubated at 37 °C for 30 minutes. Samples were boiled for 10 minutes and 8-OH-dG levels were

measured in a volume containing 30 μg of initial DNA by ELISA assay (Cayman, Ann Arbor, MI). Measures were performed in triplicate. The absorbance was measured at 420 nm (Perkin-Elmer spectrometer, Monza, Italy) according to manufacturing instructions. The level of DNA oxidation was expressed as pg/mL of 8-OH-dG.

SH-SY5Y cells or astrocytes were plated in six-well plates ($5 \cdot 10^5$/well) and grown until confluent. DNA oxidation was measured after 16 h incubation with 10 μM oxaliplatin in presence or absence of 10 μM silibinin or α-tocopherol. One hundred micrograms of Trizol-extracted DNA for each sample was digested for 2 h (37 °C) in 25 mM CH3COONa buffer, pH 4.8, containing 1 mM $ZnCl_2$ and 1.1 U of nuclease P1 (Sigma–Aldrich). The pH was then adjusted to 8.0 using 1.5 M Tris. One unit of alkaline phosphatase (Sigma–Aldrich) was added and incubated at 37 °C for 30 min. Samples were processed similarly to the tissue homogenate samples.

3.12 Cell viability assay

Cell viability was evaluated by the reduction of 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. SH-SY5Y cells (10^4 cells/wells), HT-29 cells ($5 \cdot 10^3$ cells/wells), or primary astrocytes (10^4 cells/wells), were plated into 96-well cell culture plates, grown until confluent, and treated for 24 and 48 h with various concentrations of oxaliplatin in DMEM. After extensive washing, 1 mg/ml MTT was added into each well and incubated for 45 min at 37 °C. After washing, the formazan crystals were dissolved in 100 μl dimethyl sulfoxide. The absorbance was measured at 580 nm. Experiments were performed in quadruplicate on at least three different cell batches.

3.13 Reactive oxygen species (ROS) production evaluated by dichlorofluorescein

SH-SY5Y were plated ($5 \cdot 10^4$) on 2-cm-diameter glasses for 48 h and then incubated with the organic dye 2′,7′-dichlorofluorescein (DCF; 4 μM) for 45min. Fluorescence was measured with 490 nm excitation/510 nm emission wavelengths. Oxaliplatin (30 μM) was added soon after recording basal fluorescence level (formally time 0). Fluorescence values were expressed as arbitrary units.

3.14 Superoxide dismutase (SOD)-inhibitable superoxide anion ($O_2^{\cdot-}$) production evaluated by cytochrome C assay

SH-SY5Y cells, astrocytes HT-29 cells were plated in six-well plates ($5 \cdot 10^5$ cells/well for SH-SY5Y cells and astrocytes, $3\,10^5$ cells/well for HT-29 cells) and after 48 h were incubated with or without 100 μM oxaliplatin in serum-free DMEM containing cytochrome C (1 mg/ml) for 4 h at 37 °C, in the absence or presence of 10 μM silibinin or α-tocopherol. Nonspecific cytochrome C reduction was evaluated by carrying out tests in the presence of bovine SOD (300 mU/ml). The supernatants were collected, and the optical density was spectrophotometrically measured at 550 nm. After the nonspecific absorbance was subtracted, the SOD-inhibitable $O_2^{\cdot-}$ amount was calculated by using an extinction coefficient of $2.1\ 10^4\ M^{-1} \cdot cm^{-1}$ and expressed as μM/mg protein/4 h. The 4-h incubation interval was chosen on the basis of preliminary experiments, which showed poor reliability for longer cytochrome C exposure to the cellular environment.

3.15 Caspase 3 activity

Astrocytes or HT-29 cells were plated in 6-well plates ($5 \cdot 10^5$ cells/well and $3 \cdot 10^5$ cells/ well for astrocytes and HT-29 cells respectively) and after 48 h were incubated with 100 μM oxaliplatin in presence or absence of 10 μM silibinin or α-tocopherol for 4 and 8 h. After treatment the cells were scraped in 100 μL lysis buffer (200 mM Tris–HCl buffer, pH 7.5, containing 2 M NaCl, 20 mM EDTA, and 0.2% Triton X-100). Fifty microliters of the supernatant was incubated with 25 μM fluorogenic peptide caspase substrate rhodamine 110 bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Molecular Probes) at 25 °C for 30 min. The amount of cleaved substrate in each sample was measured in a 96-well plate fluorescence spectrometer (PerkinElmer; excitation at 496 nm and emission at 520 nm). Each sample value was normalized with the protein sample concentration, using BCA assay.

3.16 Caspase 8 activity

Astrocytes, HT-29 or PC12 cells were plated in 6-well plates ($5 \cdot 10^5$ cells/well and $3 \cdot 10^5$ cells/ well for astrocytes and HT-29 or PC12 cells respectively) and after 48 h were incubated with 100 μM oxaliplatin for 8 h. After treatment the cells were scraped

in lysis buffer as suggested by the manufacturer (Molecular Probes). Fifty microliters of the supernatant was incubated with 25 μM fluorogenic peptide caspase substrate (Ac-Ile-Glu-Thr-Asp-7amino-4-trifluormethyl coumarin; Molecular Probes) at 25 °C for 30 min. The amount of cleaved substrate in each sample was measured in a 96-well plate fluorescence spectrometer (Flexi Station III; excitation at 400 nm and emission at 505 nm). Each sample value was normalized with the protein sample concentration, using BCA assay.

3.17 Cytochrome C cytosolic release evaluated with immunocytochemistry

Astrocytes or HT-29 cells were plated in D-polylisinated slides (10^5 cells/slides) and after 48 h were incubated with 100 μM oxaliplatin for 8 h. After treatment the cells were fixed with 4% paraformaldehyde in PBS, permeabilized for 10 min with PBS containing 0.1 % Triton X-100, blocked with PBS containing albumin 1 % (Sigma) and incubated overnight with a rabbit anti-cytochrome C antibody at 4 °C (1:300, Santa Cruz Biotechnology). Slides were washed three times with PBS and incubated with the appropriate secondary antibody labeled with Alexa Fluor 488 (1:500; Invitrogen) at room temperature for 1 hour. Images obtained from different slides were captured under a fluorescent microscope and the signal intensity was analyzed by FIJI software (distributed by ImageJ software). Results are expressed in percentage fixing the mean of control sample to 100%.

3.18 Western blot analysis

Astrocytes or HT-29 cells were plated in 25-cm^2 cell culture flasks (10^6 cells/flask) after 48 h were incubated with 100 μM oxaliplatin for 8 h. After treatment the cells were were homogenized in lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, Complete Protease Inhibitor (Roche), and the homogenate was incubated on ice for 30 minutes. Then, the suspension was sonicated on ice for 15 second. After centrifugation (13000×g for 15 minutes at 4°C) aliquots containing 35 μg total protein were separated on a 4–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel by electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in PBST and then probed overnight with specific primary antibody (see the table below). Membranes

were revealed with ECL assay (see Carbonylated Protein Evaluation). Densitometric analysis was performed using the "ImageJ" analysis software, and results were normalized to GAPDH immunoreactivity (1: 1000 rabbit antiserum, Cell Signalling) as internal control.

Target	Diluition	Species	Manufacturer
DR5 receptor	1:500	goat	Santa Cruz Biotechnology
Bid	1:1000	rabbit	Cell Signalling
Bcl-2	1:1000	rabbit	Cell Signalling

3.19 Statistic Analysis

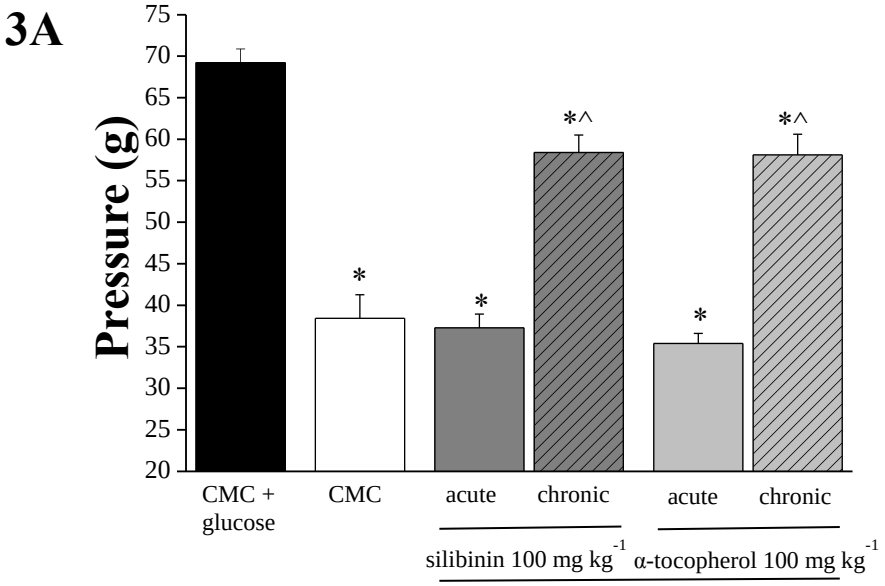
Results were expressed as means ± S.E.M. and the analysis of variance was performed by analysis of variance. A Bonferroni's significant difference procedure was used as post hoc comparison. P values of less than 0.05 or 0.01 were considered significant. Data were analyzed using the ''Origin 8.1'' software.

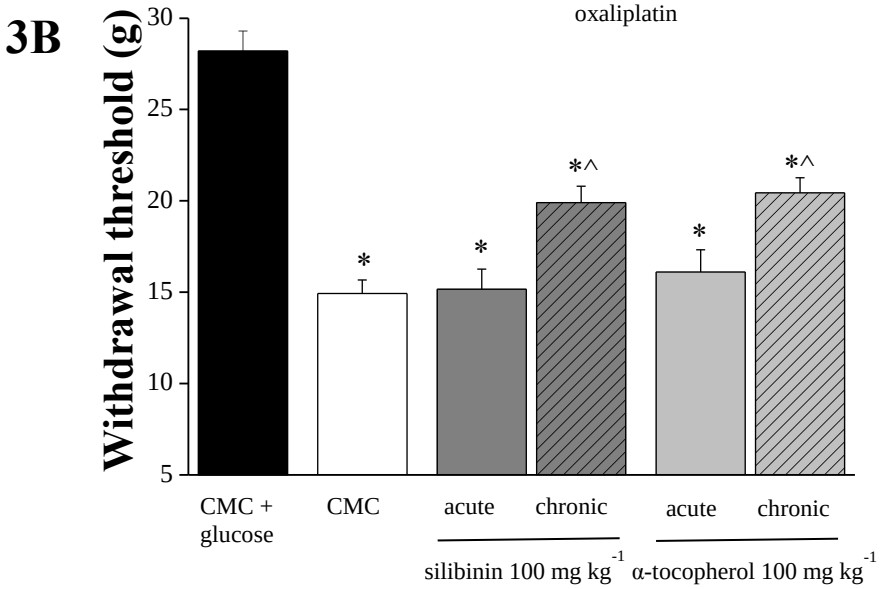
4.Results

4.1 Oxaliplatin neuropathic syndrome and the antineuropathic profile of silibin and α-tocopherol

Intraperitoneal daily administration of oxaliplatin (2.4 mg kg^{-1}) induced a neuropathy characterized by pain starting from the 14th day (data not shown). On the 21st day, the response to a noxious mechanical stimulus was increased and reached the maximum: the pressure tolerated on the posterior paw, measured by paw-pressure test, significantly decreased from the control value of 69.2 ± 1.7 g (Fig 3A, CMC + glucose) to 38.4 ± 2.8 g for the oxaliplatin-treated animals (CMC + oxaliplatin). Silibinin and α-tocopherol-treated animals (100 mg·kg^{-1}p.o. administered daily from day 1 to day 20) showed a significant lower responsiveness to a noxious stimulus (57.3 ± 1.6 g and 58.4 ± 2.1 g, respectively; Fig 3A, chronic) in respect to the oxaliplatin group. Both molecules were ineffective when administered once, 30 minutes before test (Fig 3A, acute). Moreover, oxaliplatin induced a lowered threshold to mechanical and cold stimuli which do not normally provoke pain (Figs 3B and 3C). As measured with the electronic Von Frey apparatus, control withdrawal threshold to the non-noxious mechanical stimulus was decreased in oxaliplatin treated animals from 28.2 ± 1.1 g to 14.9 ± 1.6 g (Fig 3B). Repeated silibinin administration partially modified pain threshold increasing the tolerated pressure on the anterior paw up to 19.9 ± 0.9 g. A comparable effect was induced by α-tocopherol (Fig 3B, chronic). The sensibility to a cold surface is depicted in Fig 3C. After oxaliplatin treatment, the licking latency decreased from 25.1 ± 1.1 s (CMC + glucose) to 14.2 ± 0.4 s (CMC + oxaliplatin). Both antioxidants tested significantly prevented cold hypersensibility (silibinin: 19.4 ± 0.5 s; α-tocopherol: 19.7 ± 0.2 s). In both Von Frey and cold plate test, silibinin and α-tocopherol acutely administered before test were ineffective. Chemotherapy-induced neuropathy impaired motor coordination as evaluated by the walking time on a rotating rod and the number of falls (Rota-rod test). Indeed, control rats were able to balance on the rotating rod for 600 seconds (Fig 4A), falling down 0.6 ± 0.1 folds (Fig. 2B). On the contrary, oxaliplatin treated animals maintained the balance for 163 ± 67 seconds only and fell down 4.9 ± 0.2 times (Fig 4A and 4B). Silibinin and α-tocopherol improved the time of walking up to 415 ± 64 seconds and 409 ± 80 seconds respectively (Fig 4A and 4B, chronic) but they were ineffective on

the number of falls. Again, antioxidant acute treatment was ineffective (Fig 4A and 4B, acute).





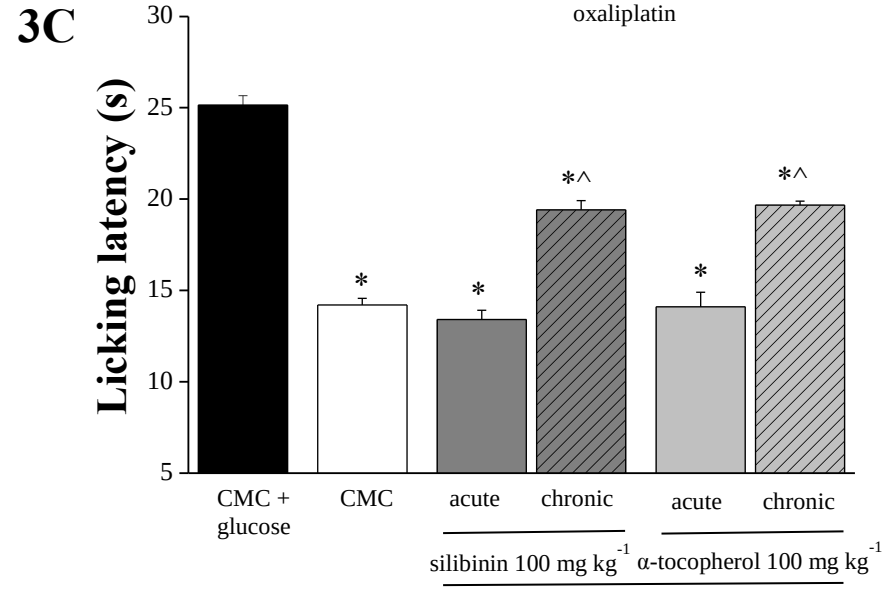
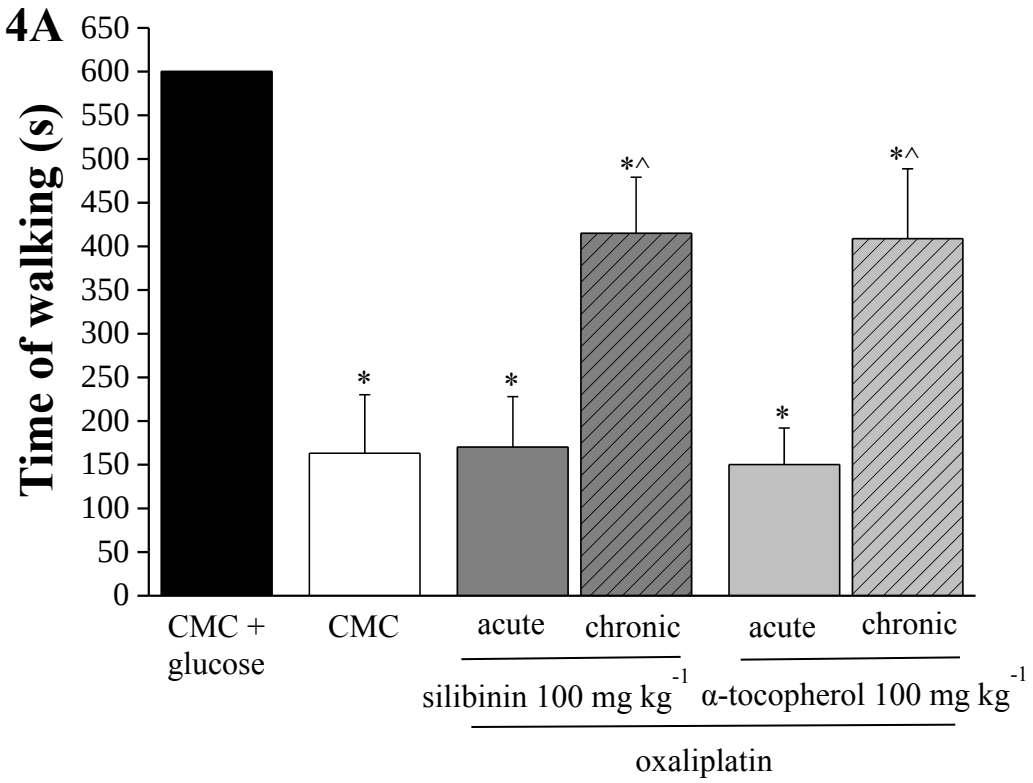


Figure 3. Pain perception in oxaliplatin-treated rats**. A**. Pain: noxious stimulus, Paw-pressure test. Paw pressure test was used to measured hyperalgesia. **B**. Pain: non-noxious mechanical stimulus. Von Frey test was used to measure the response evoked by a mechanical stimulus. **C**. Pain: non-noxious thermal stimuli. The response to a thermal stimulus was evaluated by cold plate test measuring the latency to pain-related behaviors (lifting or licking of the paw).

For each tests rats were daily intraperitoneally treated with 2.4 mg kg^{-1} oxaliplatin (dissolved in 5% glucose). Behavior was evaluated on day 21. Silibinin or α-tocopherol (100 $mgkg^{-1}$, dissolved in CMC) were per os administered acutely (single injection 30 minutes before the test; acute) or repetitively (daily for 20 days starting from the first day of oxaliplatin administration; chronic). Control animals were treated with vehicles. Each value represents the mean of 24 rats per group, performed in 2 different experimental set. *P < 0.01 versus CMC + glucose (control). ^P < 0.01 versus CMC + oxaliplatin.


4A
Time of walking (s)
650
600
550
500
450
400
350
300
250
200
150
100
50
0
*
*
*^
*
*^
CMC + glucose
CMC
acute
chronic
acute
chronic
silibinin 100 mg kg-1
α-tocopherol 100 mg kg-1
oxaliplatin

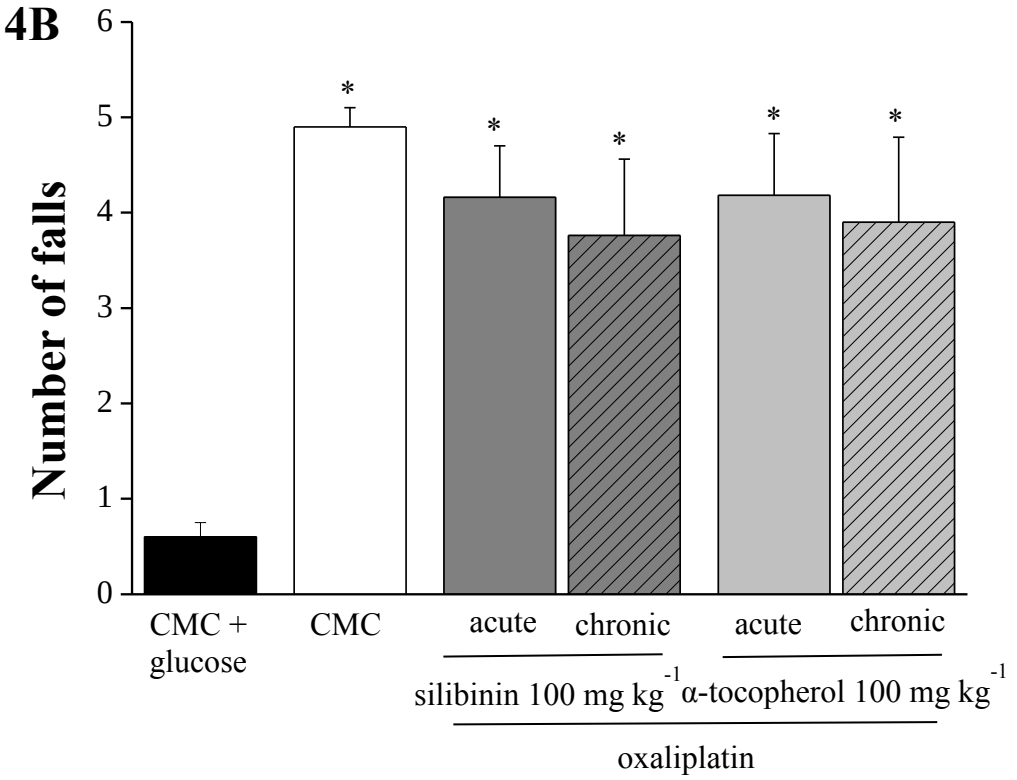

4B
Number of falls
6
5
4
3
2
1
0
*
*
*
*
*
CMC + glucose
CMC
acute
chronic
acute
chronic
silibinin 100 mg kg-1
α-tocopherol 100 mg kg-1
oxaliplatin

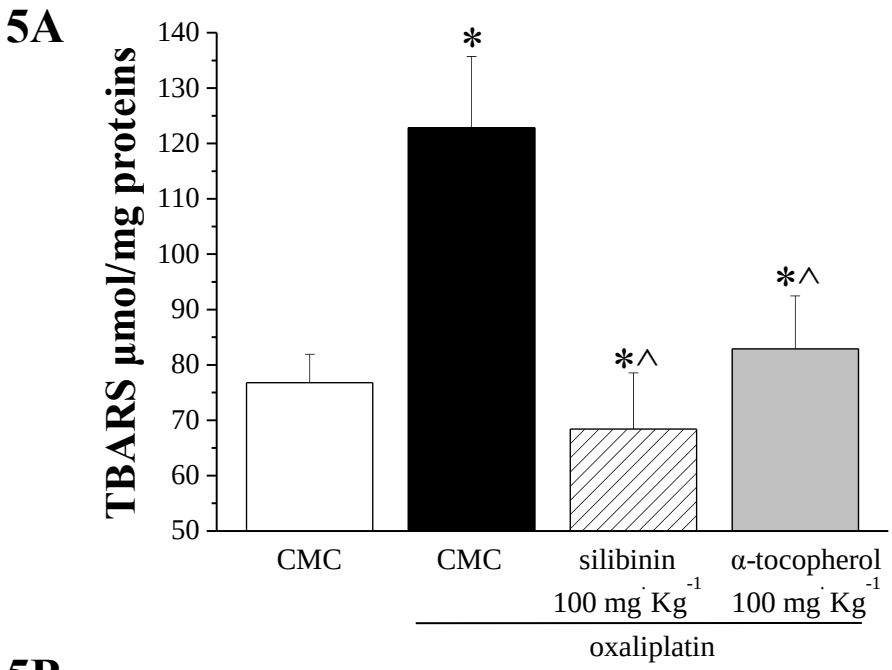
Figure 4. Motor coordination in oxaliplatin-treated rats. The integrity of the animals' motor coordination was assessed using a rota-rod apparatus. Rats were placed on a rotating rod (10 rpm) for a maximum of 10 minutes (600 s). The time spent in the balance (**A**) and the number of falls (**B**) was counted. Rats were daily intraperitoneally treated with 2.4 mg kg^{-1} oxaliplatin (dissolved in 5% glucose). Motor coordination was evaluated at day 21. Silibinin or α-tocopherol (100 mg kg^{-1}, dissolved in CMC) were per os administered acutely (single injection 30 minutes before the test; acute) or repetitively (daily for 20 days starting from the first day of oxaliplatin administration; chronic). Control animals were treated with vehicles. Each value represents the mean of 24 rats per group, performed in 2 different experimental set. *P < 0.01 versus CMC + glucose (control). ^P < 0.01 versus CMC + oxaliplatin.

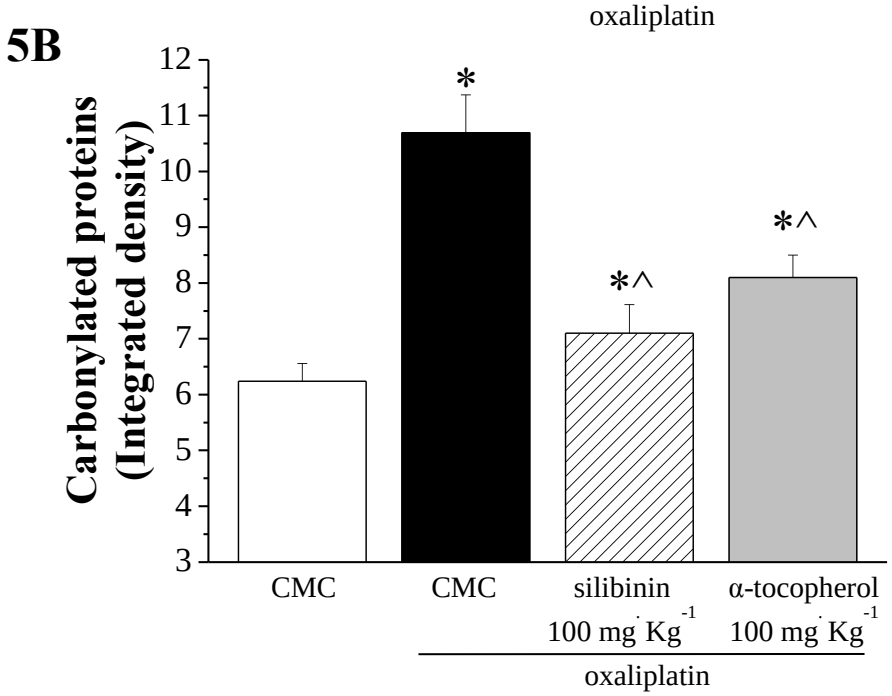
4.2 Oxidative damage induced by oxaliplatin and the preventive effects of silibinin and α-tocopherol

Aimed to evaluate the oxidative stress when the neuropathic syndrome is clearly established, rat plasma samples were analyzed on day 21 measuring TBARS and protein carbonylation. In plasma of oxaliplatin treated rats, lipid peroxidation was significantly increased as compared with control animals (CMC + glucose). The increase in TBARS induced by oxaliplatin treatment was fully prevented by silibinin and α-tocopherol repeated treatment (Fig 5A). Carbonylated protein levels were about twice in oxaliplatin-treated rats than in control animals (Fig 5B). Protein oxidation was prevented by 81 % and 58% by silibinin and α-tocopherol, respectively (Fig 5B).

The study of the oxidative damage evoked by oxaliplatin was extended to the nervous system, final target of chemotherapy toxicity. Both peripheral and central nervous system of 21 day-treated rats showed an increase in lipid peroxidation as reported in Fig 4A. Sciatic nerve TBARS levels increased in the oxaliplatin group from 38.4 ± 5.3 to 57.6 ± 3.0 mmol/mg proteins (Fig 6A, left panel) and in spinal cord from 94.7 ± 12.2 to 179.8 ± 24.3 (Fig 6A, right panel). Antioxidants exerted a preventive effect of about 80% in the sciatic nerve and 70% in the spinal cord. Moreover, oxidative stress at protein level was highlighted in both sciatic nerve and spinal cord of oxaliplatin-treated animals by a 3x increase in carbonylated level in respect to control. Silibinin

and α-tocopherol inhibited protein oxidation by about 60% (Fig 6B). Finally, DNA oxidation was evaluated in nervous tissue. In the sciatic nerve, the basal level of 8-OH-dG was 3.2 ± 2.3 pg/mL (CMC + glucose). Oxaliplatin increased this value up to 38.6 ± 8.3 pg/mL; silibinin and α-tocopherol decreased oxaliplatin-induced DNA oxidation by 48% and by 63% respectively (7A, left panel). Also in the spinal cord, oxaliplatin increased 8-OH-dG from 8.7 ± 2.2 pg/mL (CMC + glucose) to 52.8 ± 4.3 pg/mL (CMC + oxaliplatin); silibinin and α-tocopherol prevented 8-OH-dG formation by 65 and 76% respectively (Fig 7B).


5A
TBARS µmol/mg proteins
140
130
120
110
100
90
80
70
60
50
*
*^
*^
CMC
CMC
silibinin
100 mg Kg-1
α-tocopherol
100 mg Kg-1
oxaliplatin


5B
Carbonylated proteins
(Integrated density)
12
11
10
9
8
7
6
5
4
3
*
*^
*^
CMC
CMC
silibinin
100 mg Kg-1
α-tocopherol
100 mg Kg-1
oxaliplatin

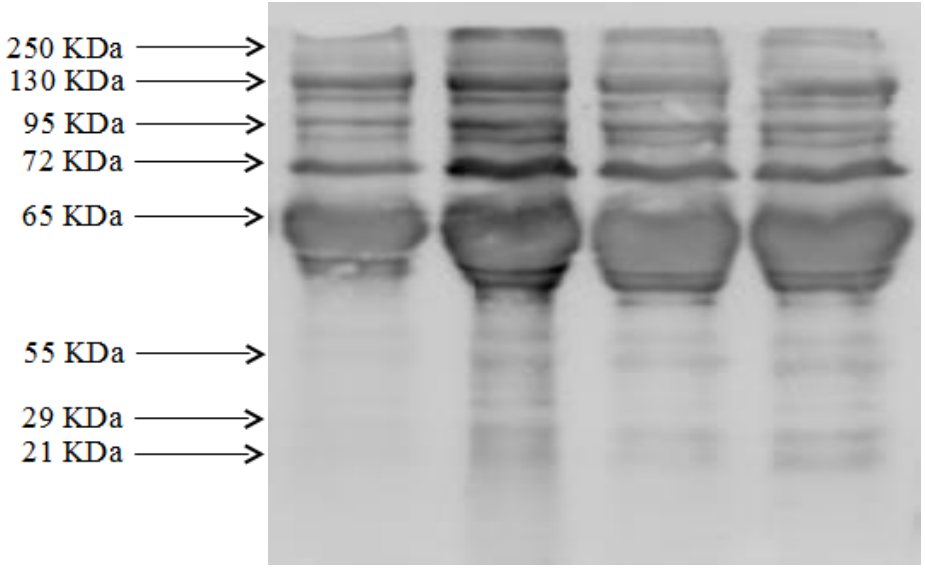
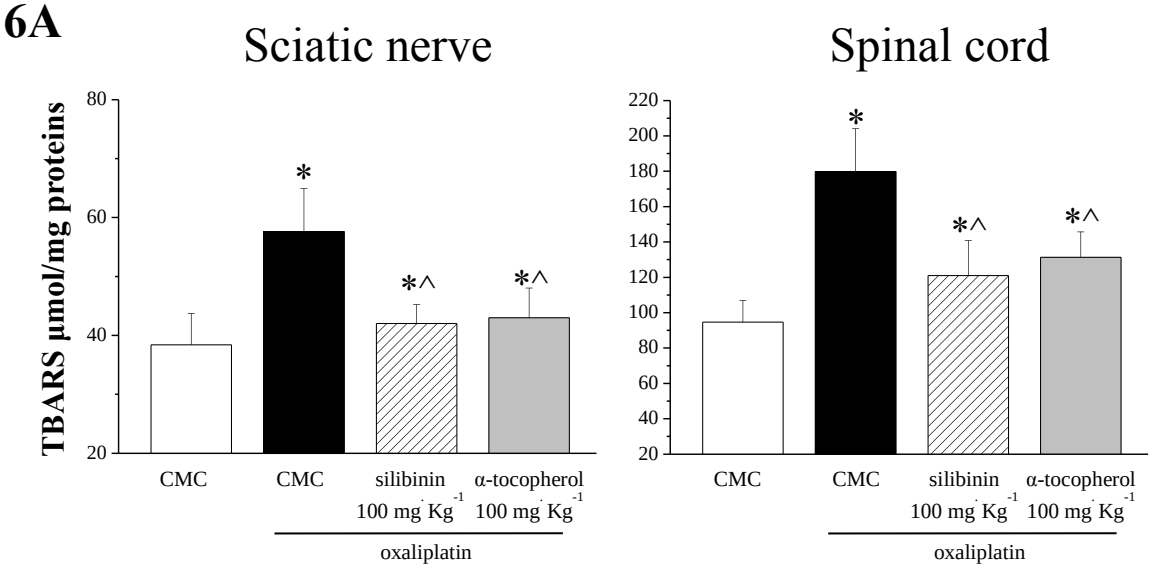

250 KDa
130 KDa
95 KDa
72 KDa
65 KDa
55 KDa
29 KDa
21 KDa

Figure 5. Plasma oxidation levels in rats treated with oxaliplatin. At day 21, plasma was collected and oxidative stress evaluated. **A** Lipid peroxidation was evaluated measuring TBARS levels. **B,** Protein oxidative damage was measured quantifying carbonylated proteins by immunoblot. Densitometric analysis (top panel) and representative Western blot (lower panel) are shown. Ponceau-stained membranes were used as loading control.

For each mesure silibinin or α-tocopherol (100 mg kg^{-1}, dissolved in CMC) were per os repetitively administered (daily for 20 days starting from the first day of oxaliplatin administration). Control animals were treated with vehicles. Each value represents the mean of 12 rats per group, performed in 2 different experimental set. *P < 0.01 versus CMC + glucose (control). ^P < 0.01 versus CMC + oxaliplatin.

6A


Sciatic nerve
Spinal cord
TBARS µmol/mg proteins
CMC
CMC
silibinin 100 mg Kg-1
α-tocopherol 100 mg Kg-1
oxaliplatin

6B

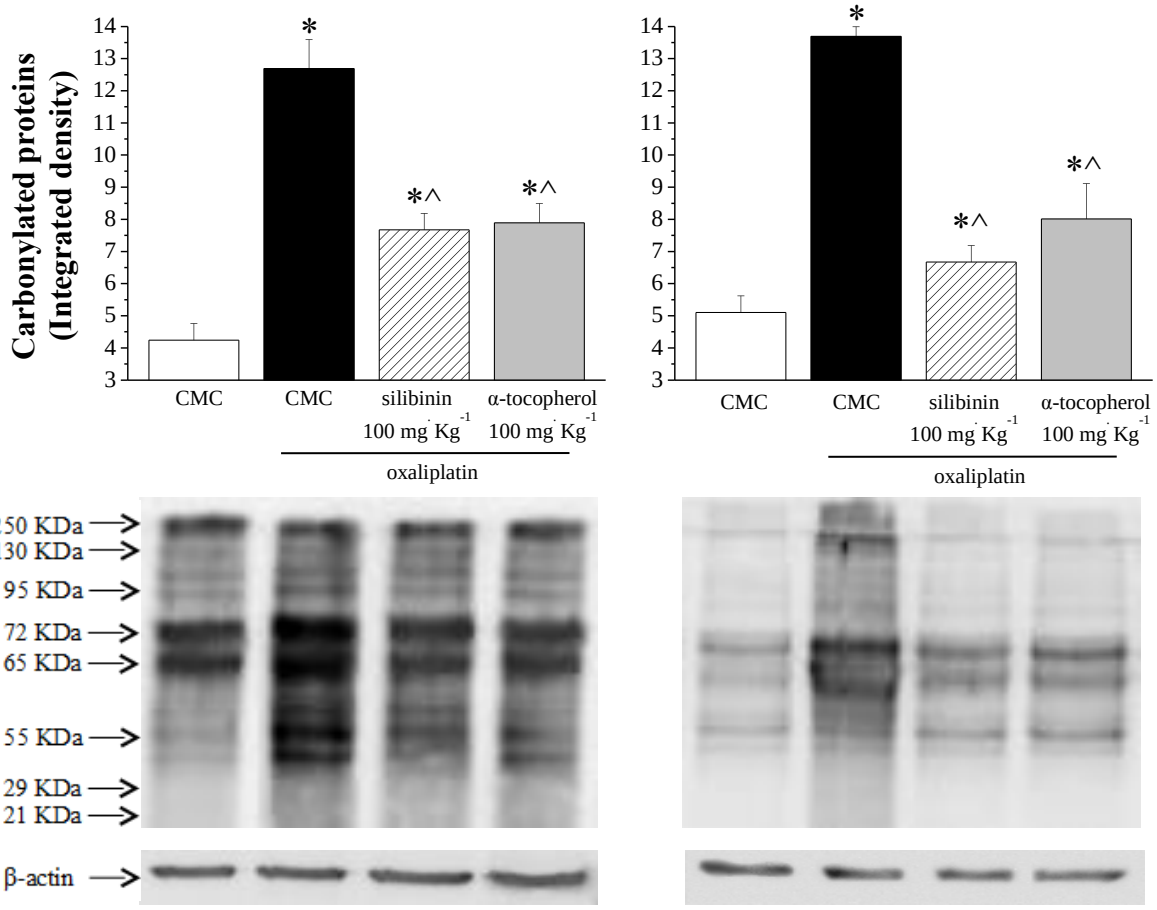

Carbonylated proteins (Integrated density)
CMC
CMC
silibinin 100 mg Kg-1
α-tocopherol 100 mg Kg-1
oxaliplatin
250 KDa
130 KDa
95 KDa
72 KDa
65 KDa
55 KDa
29 KDa
21 KDa
β-actin

Figure 6. Nervous tissue oxidation levels in rats treated with oxaliplatin. At 21st day, the sciatic nerve and the lumbar tract of the spinal cord were explanted. Peripheral and central nervous tissues were analyzed for both lipid and protein oxidation. **A.** TBARS levels in sciatic nerve (left panel) and in spinal cord (right panel). **B.** Carbonylated protein in sciatic nerve (left panel) and in spinal cord (right panel). Densitometric analysis (top panel) and representative Western blot (lower panel) are shown. β-actin normalization was performed for each sample. Silibinin or α-tocopherol (100 mg kg^{-1}, dissolved in CMC) were per os repetitively administered (daily for 20 days starting from the first day of oxaliplatin administration). Control animals were treated with vehicles. Each value represents the mean of 12 rats per group, performed in 2 different experimental set. *P < 0.01 versus CMC + glucose (control). ^P < 0.01 versus CMC + oxaliplatin.

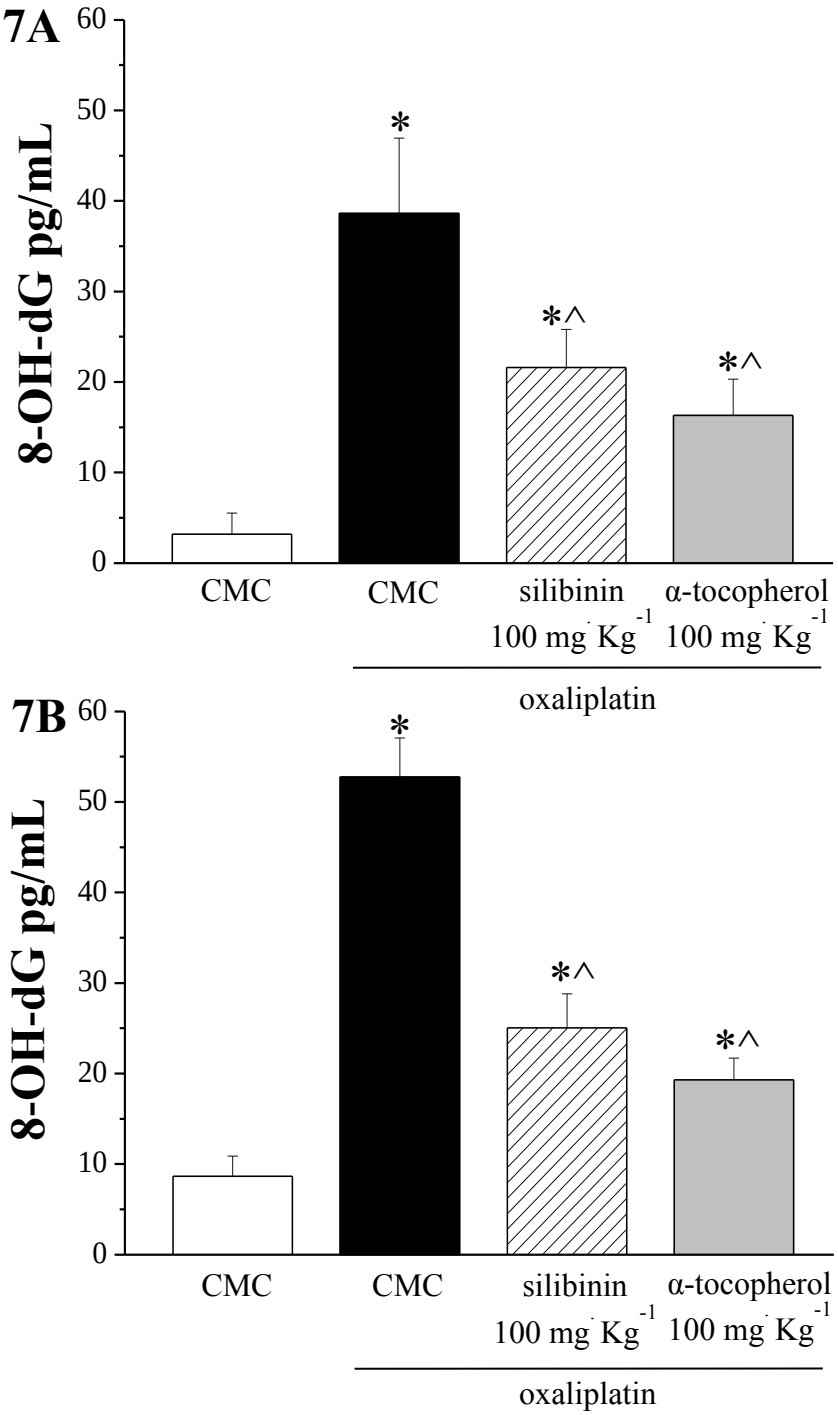


Figure 7. DNA oxidation levels in sciatic nerve (**A**) and spinal cord (**B**) of oxaliplatin-treated rats. DNA was extracted from sciatic nerve and spinal cord of treated animals at day 21. DNA samples were analyzed by ELISA method to quantify 8-OH-2-dG levels. Silibinin or α-tocopherol (100 mg kg^{-1}, dissolved in CMC) were per os repetitively administered (daily for 20 days starting from the first day of oxaliplatin administration). Control animals were treated with vehicles. Each value represents the

mean of 12 rats per group, performed in 2 different experimental sets. *P < 0.01 versus CMC + glucose (control). ^P < 0.01 versus CMC + oxaliplatin.

4.3 Cellular model of oxaliplatin neurotoxicity

4.3.1 SH-SY5Y cell line

Cell viability after oxaliplatin treatment

According to the aim, our research started by analyzing the effect of oxaliplatin on the neuroblastoma cell line SH-SY5Y. In this cell line, oxaliplatin decreased cell viability in a concentration-dependent way (Fig. 8). Fifty percent lethal concentration (LC_{50}) was 2.7 ± 0.6 and 1.0 ± 0.1 µM after 24 and 48 h, respectively.

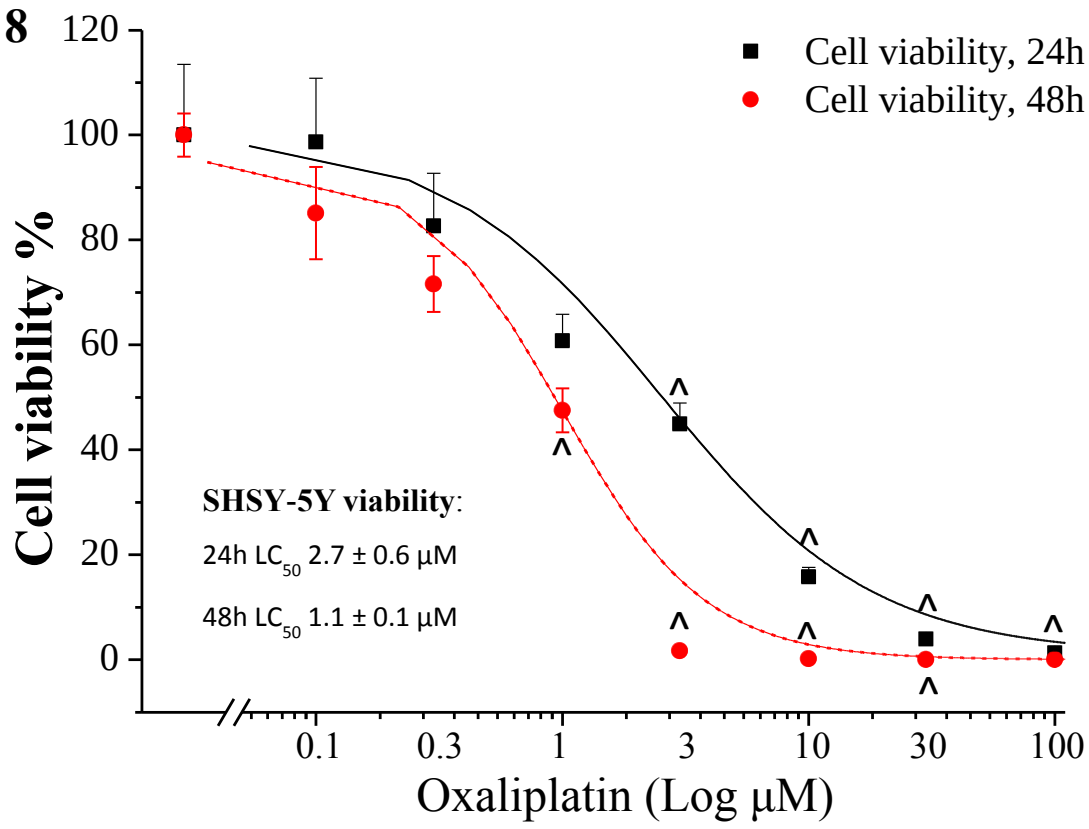


Figure 8. SH-SY5Y cells. Cell viability. SH-SY5Y (10^4 cells/well) were incubated with oxaliplatin (0.1–100 µM) for 24 or 48 h. Viability was quantified by MTT assay; absorbance was measured at 550 nm. Values are expressed in percentage of control

absorbance as the mean ± SEM of six experiments. Control condition absorbance was fixed at 100%. ^P < 0.01 vs control.

Oxidative damage after oxaliplatin treatment

In DCF-loaded cells, 30 μM oxaliplatin induced a time-dependent ROS production (Fig. 9). Aimed to evaluate the effects of oxaliplatin-induced oxidative stress, lipid, protein, and DNA damage was studied after 16 h incubation with 10 μM oxaliplatin. Lipid peroxidation, measured as TBARS, was increased with respect to control values. In the presence of 10 μM silibinin or α-tocopherol, lipid peroxidation was inhibited by about 55% (Table 2). Oxaliplatin also induced protein oxidation as evaluated by protein carbonylation measure. The two-fold increase in protein carbonylation was prevented up to 60 and 45% by silibinin and α-tocopherol, respectively (Table 2). Finally, redox imbalance led to 6.5-fold increased 8-OH-dG; silibinin and α-tocopherol decreased oxaliplatin-induced DNA oxidation by 56 and 43% respectively (Table 2).

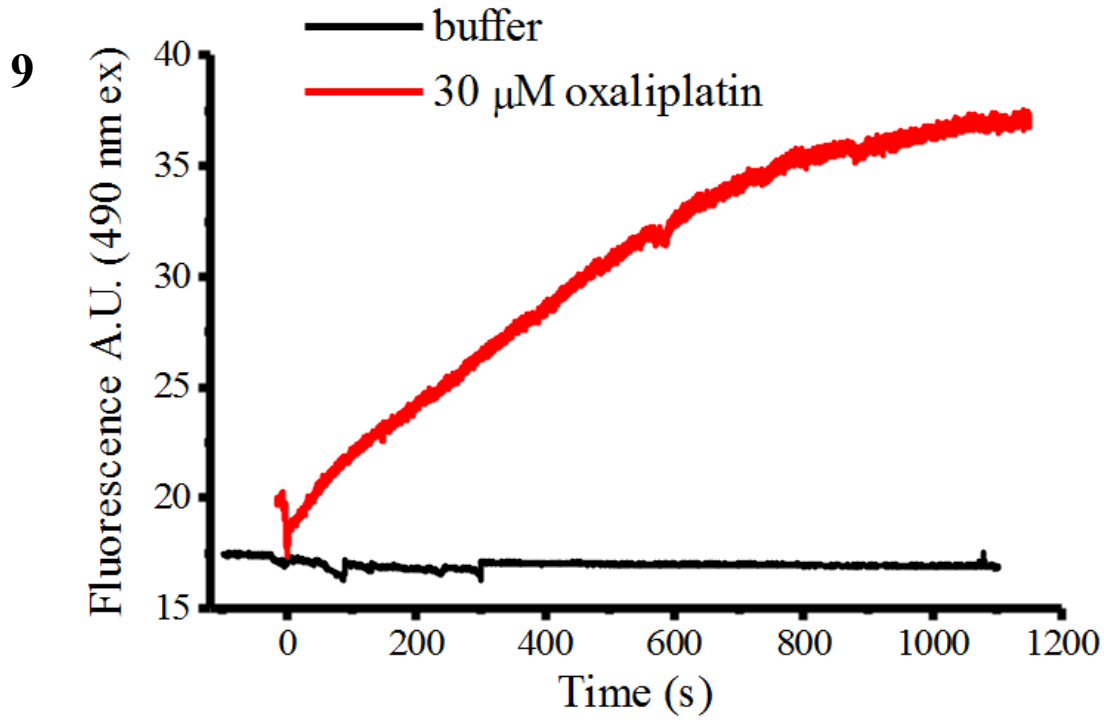


Figure 9. SH-SY5Y cells. Oxidative damage oxaliplatin-induced. ROS production evaluated by dichlorofluorescein. SH-SY5Y cells were incubated with 2′,7′-DCF (4

μM, 45 min). Fluorescence was measured at 490 nm ex/510 nm em wavelengths and reported as arbitrary units (A.U.). 30 μM oxaliplatin was added at time 0.

	control	oxaliplatin	oxaliplatin + silibinin (10 μM)	oxaliplatin + α–tocopherol (10 μM)
Lipoperoxidation (TBARS μmol/mg protein)	**19.51± 1.62**	**43.46 ± 2.43***	**29.98 ± 1.76*^**	**29.08 ± 0.97*^**
Protein carbonylation (integrated density)	**4.56 ± 0.18**	**10.2 ± 1.21***	**6.84 ± 0.45*^**	**7.69 ± 0.32*^**
DNA oxidation (8-OH-dG pg/ml)	**3.12 ± 1.14**	**20.36 ± 2.21***	**10.68 ± 2.45*^**	**12.98 ± 3.1*^**

Table 2. Effects of silibinin and α-tocopherol on oxaliplatin-induced oxidative stress in SH-SY5Y cells**.** SH-SY5Y cells were exposed to 10 μM oxaliplatin for 16 h in the presence or in the absence of silibinin or α-tocopherol (10 μM). Silibinin or α-tocopherol was co-incubated with the chemotherapeutic agent. Values are expressed as the means ± SEM of three experiments. *P<0.01 vs control and ^P<0.01 vs oxaliplatin treatment

4.3.2 Primary rat astrocytes

Cell viability after oxaliplatin treatment

In primary astrocytes, oxaliplatin decreased cell viability with LC_{50} > 100 µM and 16.0 ± 0.1 µM after 24 and 48 h, respectively (Fig. 10).

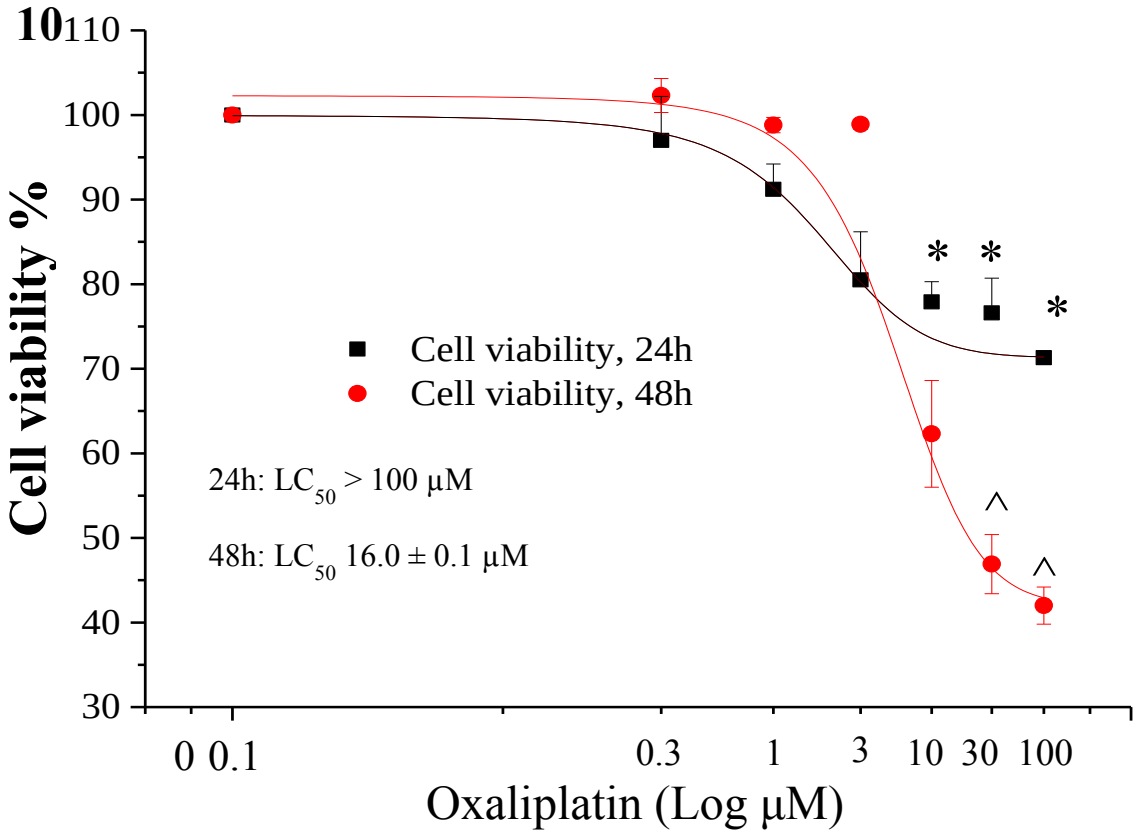


Figure 10. Astrocyte cell viability. Astrocytes (10^4 cells/well) were incubated with oxaliplatin (0.1–100 µM) for 24 or 48 h. Viability was quantified by MTT assay. Values are expressed in percentage of control absorbance as the mean ± SEM of six experiments. Control condition absorbance was fixed at 100%. *P < 0.05 and ^P < 0.01 vs control.

Oxidative damage after oxaliplatin treatment

Oxaliplatin, incubated for 16 h at 10 μM, induced lipid, protein, and DNA oxidation. In the presence of 10 μM silibinin or α-tocopherol, lipid peroxidation was inhibited by 51 and 40%, respectively (Fig. 11). In the astrocyte culture the basal level of 8-OH-dG was 5.25 ± 2.06 pg/ml. Oxaliplatin increased this value up to nine fold (48.94 ± 3.78 pg/ml); silibinin decreased oxaliplatin-induced DNA oxidation by 82%. The preventive effect of α-tocopherol was by 53% (Fig. 12A). Carbonylated protein level was about twice in oxaliplatin-treated cells compared to control samples and it was prevented by 41 and 35% by silibinin and α-tocopherol, respectively (Fig. 12B).

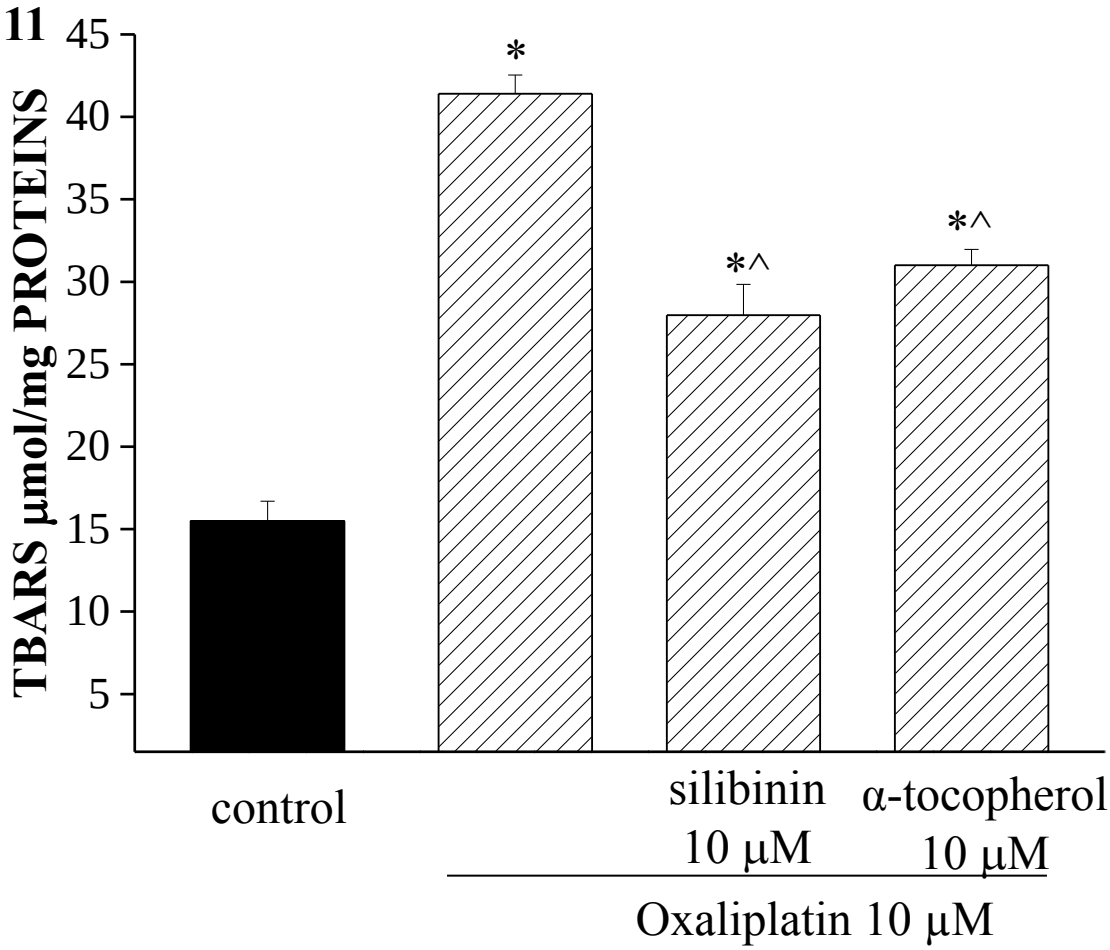
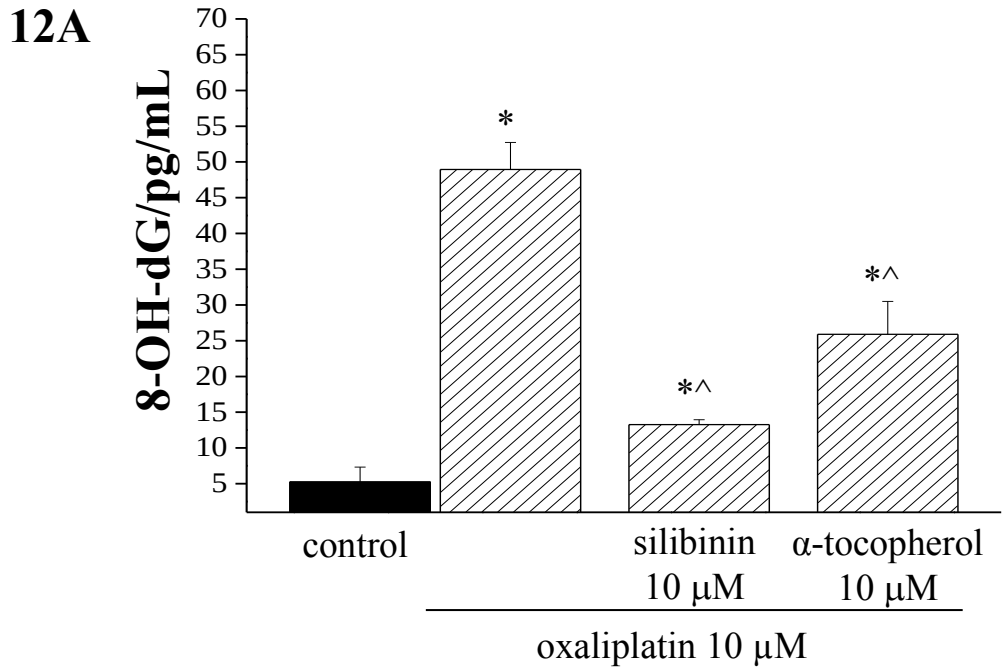


Figure 11. Astrocyte cells. Oxidative damage oxaliplatin-induced**.** Astrocytes. Lipoperoxidation. Astrocytes (10^6 cells/flask) were exposed to 10 μM oxaliplatin for 16 h in the absence or presence of silibinin or α-tocopherol (10 μM). TBARS assay

was used to evaluate lipid peroxidation. *P < 0.01 vs control and ^P < 0.01 vs oxaliplatin treatment.



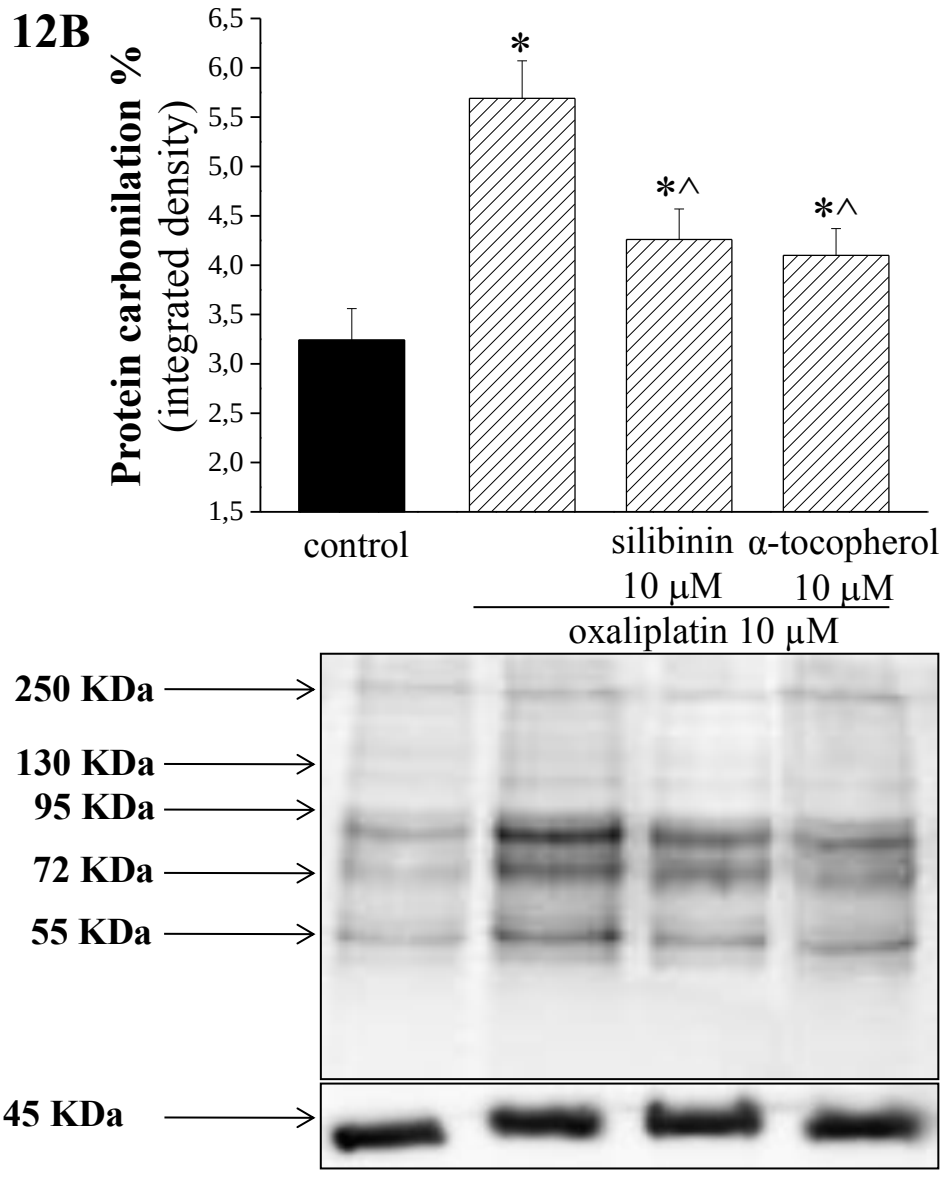
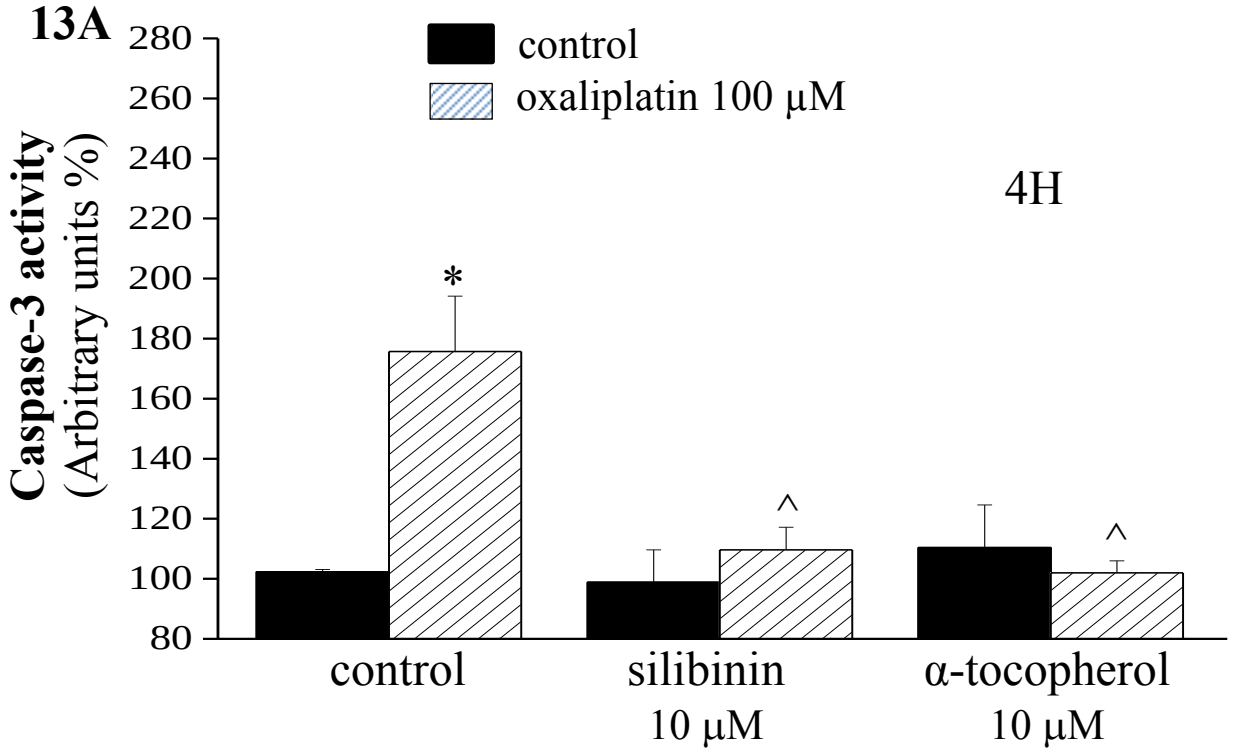


Figure 12. Astrocyte cells. Oxidative damage oxaliplatin-induced. **A.** DNA oxidation. DNA was extracted from cultures after 16 h incubation with oxaliplatin (10 μM) in the absence or presence of silibinin or α-tocopherol (10 μM). DNA samples were analyzed by ELISA to quantify 8-OH-2-dG levels. Values are expressed as the mean ± SEM of three experiments. *P < 0.01 vs control and ^ P < 0.01 vs oxaliplatin treatment. **B**. Protein carbonylation. Carbonylated proteins were measured in astrocytes treated for 16 h with 10 μM oxaliplatin in the absence or presence of silibinin or α-tocopherol (10 μM). Western blot analysis was performed on cell homogenates, using a specific antibody. Densitometric analysis (top) and representative immunoblot (bottom) are shown. Data are expressed as percentage of control. β-actin was used as loading control. *P < 0.01 vs control and ^P < 0.01 vs oxaliplatin treatment.

Caspase-3 activation after oxaliplatin treatment

Finally, the apoptotic oxaliplatin damage was evaluated. Fig. 12 shows the effect of 100 μM oxaliplatin on caspase-3 activity. Four hours incubation induce a 1.8-fold increase in the enzyme activity (Fig. 13A) that reach 2.5-fold after 8 h (Fig. 13B); 10 μM silibinin or α- tocopherol fully prevented caspase-3 activation at both times.



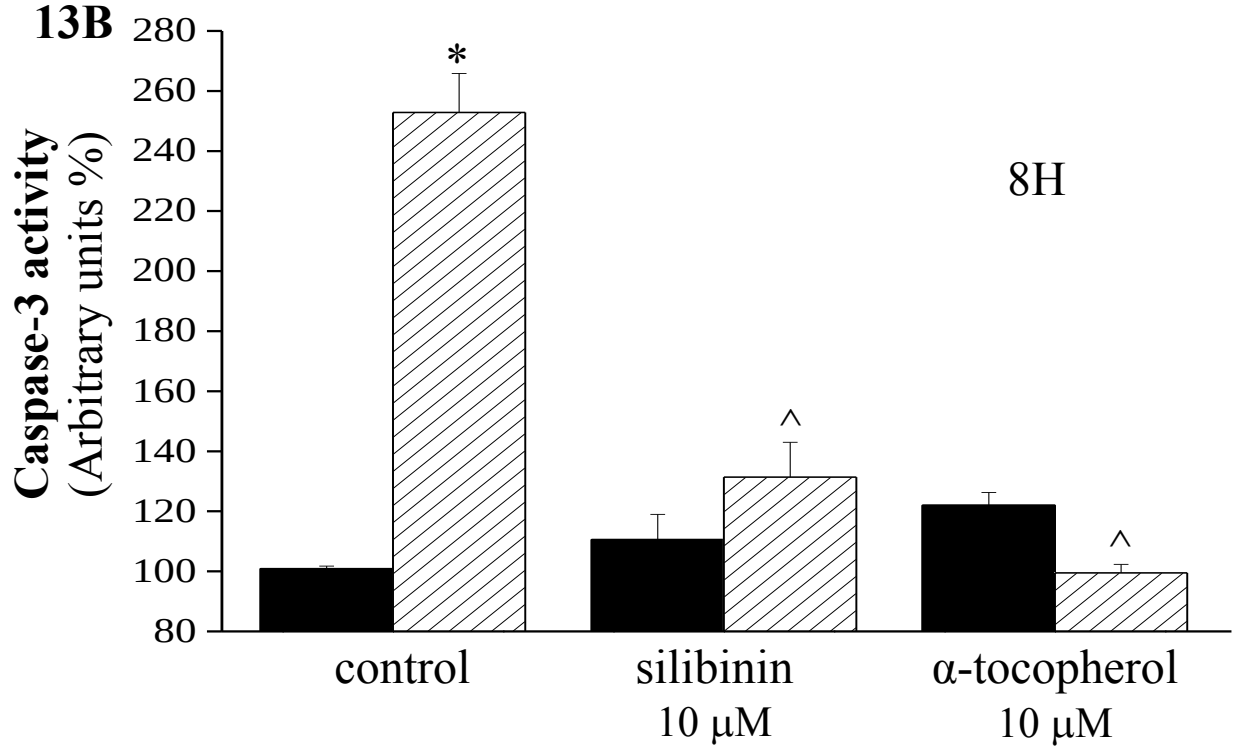


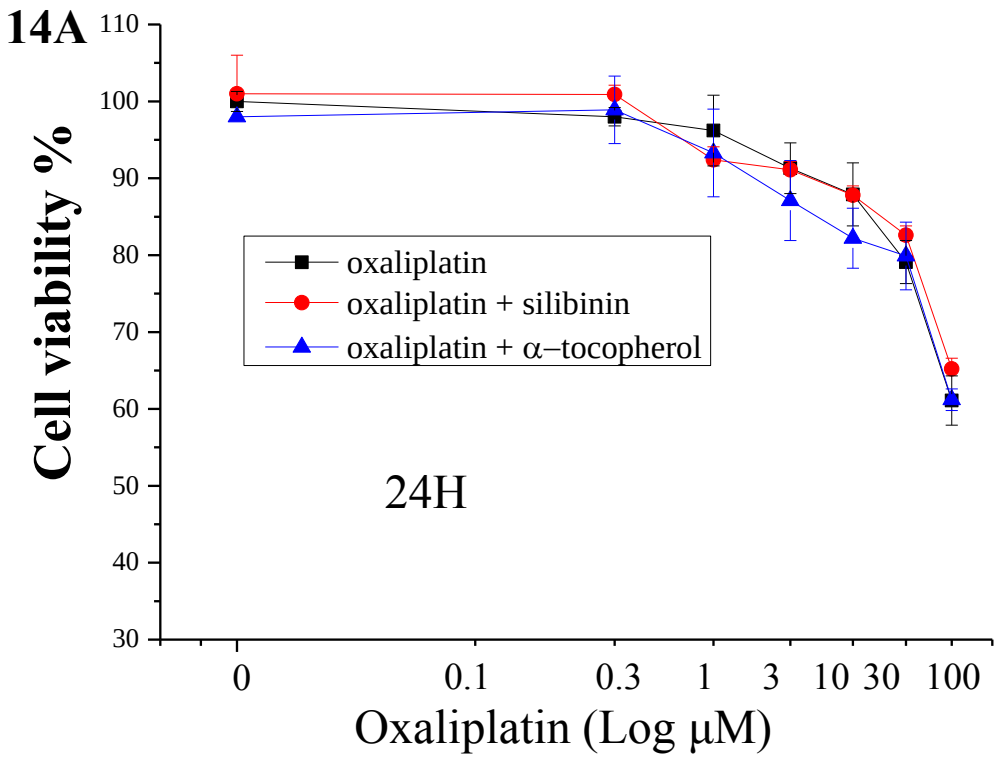
Figure 13. Caspase-3 activity in astrocytes. Cells ($5 \cdot 10^5$ cells/well) were treated with oxaliplatin (100 μM) in the absence or presence of silibinin or α- tocopherol (10 μM) and incubated for (**A**) 4 and (**B**) 8 h. Caspase-3 activity was measured by a fluorescence assay. Values are expressed as percentage of control caspase-3 activity arbitrarily set as 100%. Bars represent the mean ± SEM of three experiments. *P < 0.01 vs control and ^P < 0.01 vs oxaliplatin treatment.

4.4 Effects of the antioxidants silibinin and α-tocopherol on the oxaliplatin anticancer properties

4.4.1 HT-29 cell line

Viability and caspase-3 activity

Aimed to evaluate the interaction between the antioxidant treatment and the therapeutic property of oxaliplatin, we used a human adenocarcinoma colo-rectal cell line (HT-29 cells). Figure 14A shows the concentration-dependent (0.1–100 μM) oxaliplatin lethal effect after 24 h incubation in the absence and in the presence of the tested antioxidants. Neither 10 μM silibinin nor 10 μM α-tocopherol significantly modified oxaliplatin lethality. The oxaliplatin toxic activity on HT-29 cancer cells was also not modified in the presence of the antioxidants after 48 h incubation (Figure 14B). Studying caspase-3 activation in HT-29, oxaliplatin increased the enzyme cleavage activity by 1.5- and 1.8-fold after 4 and 8 h, respectively (Figs. 15A and 15B). In the cancer cell line, differently from astrocyte cells, neither silibinin nor α-tocopherol exerted any protective effect. Moreover, 10 μM antioxidant compound *per se* (in the absence of oxaliplatin) was able to induce an increase in caspase-3 enzymatic activity (20%) after 4 h (Fig. 15A). This direct apoptotic effect disappeared when the incubation was protracted for 8 h.


14A
Cell viability %
110
100
90
80
70
60
50
40
30
oxaliplatin
oxaliplatin + silibinin
oxaliplatin + α–tocopherol
24H
0
0.1
0.3
1
3
10
30
100
Oxaliplatin (Log μM)

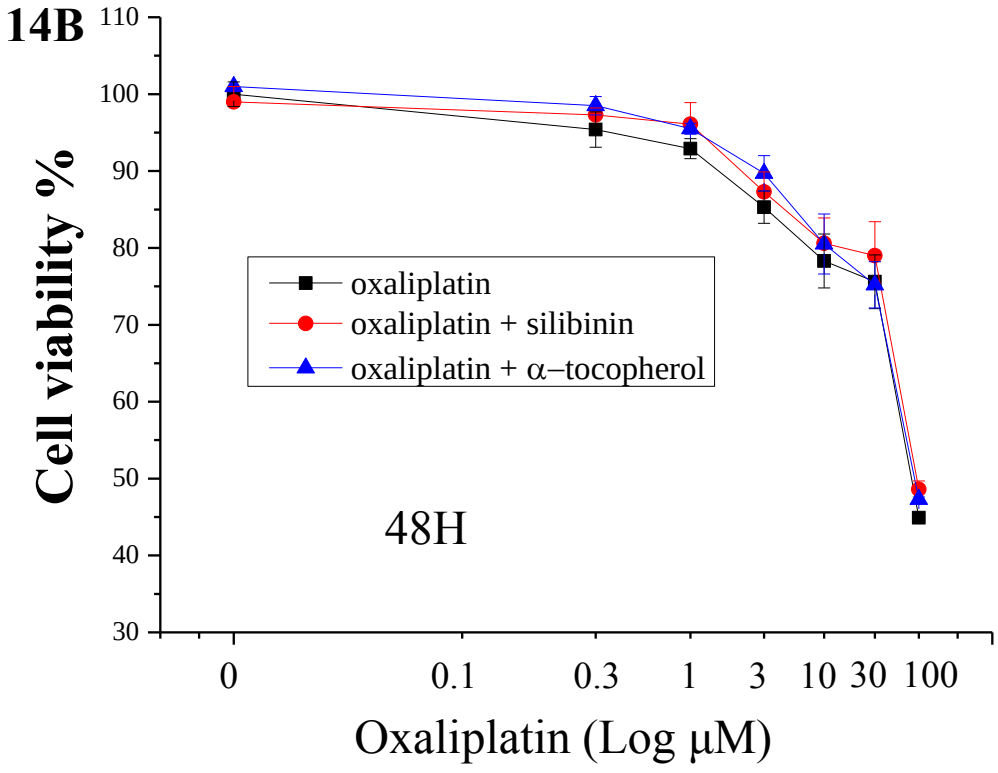

14B
Cell viability %
110
100
90
80
70
60
50
40
30
oxaliplatin
oxaliplatin + silibinin
oxaliplatin + α–tocopherol
48H
0
0.1
0.3
1
3
10
30
100
Oxaliplatin (Log μM)

Figure 14. HT-29 cell viability. HT-29 (10^4 cells/well) were incubated with oxaliplatin (0.1–100 μM) for 24 (**A**) or 48 h (**B**). Viability was quantified by MTT assay. Values are expressed in percentage of control absorbance as the mean ± SEM of six experiments. Control condition absorbance was fixed at 100%.

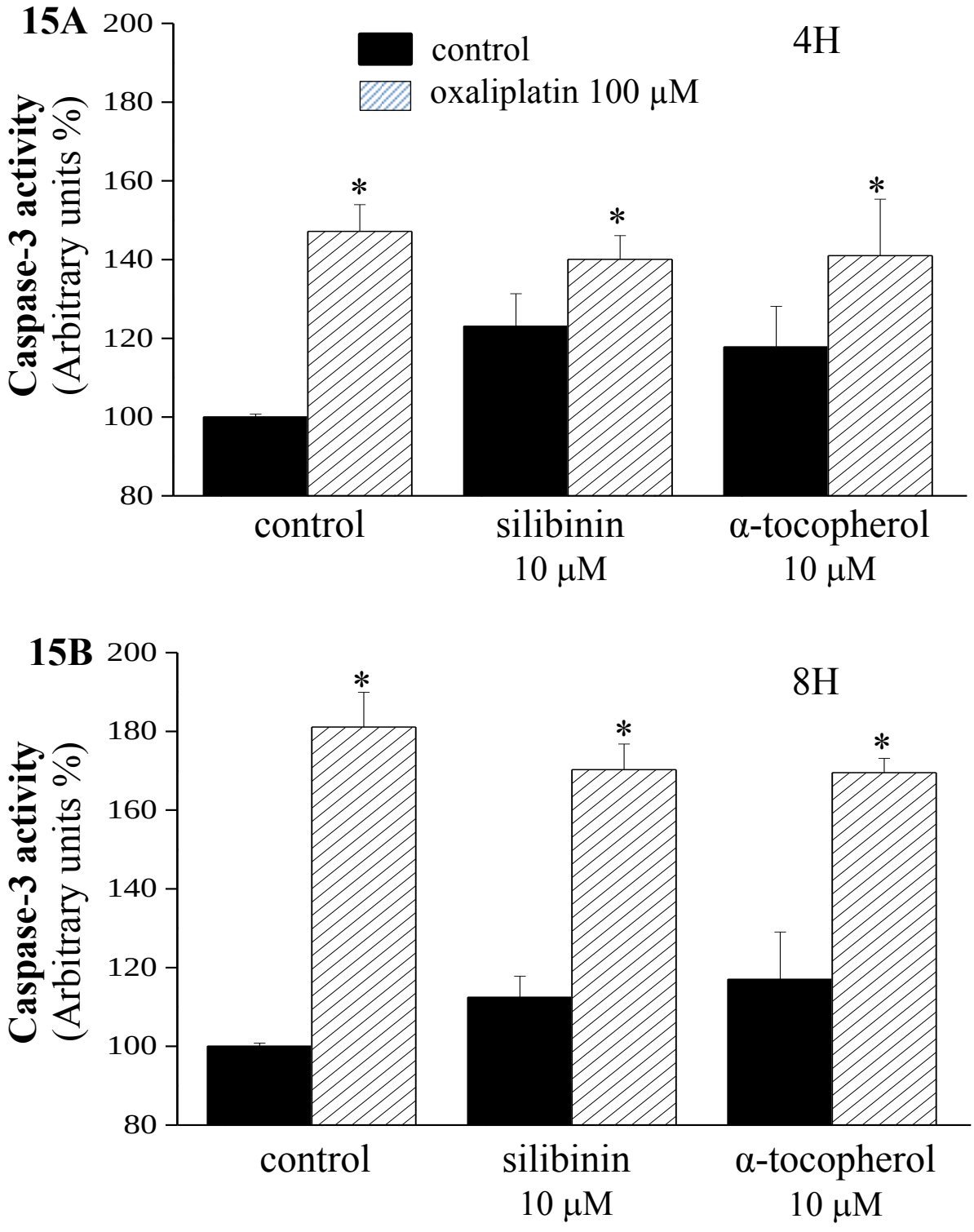
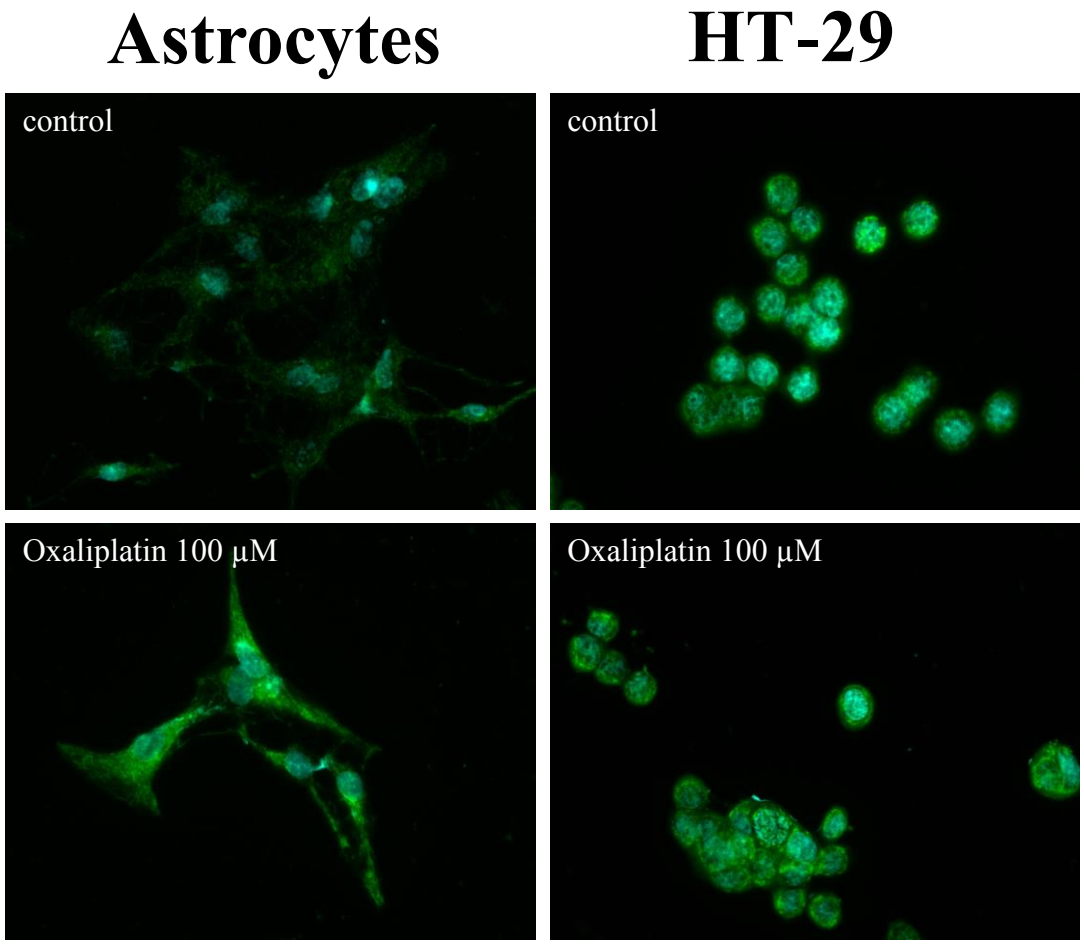


Figure 15. Caspase-3 activity in HT-29 cells. Cells ($3 \cdot 10^5$ cells/well) were treated with oxaliplatin (100 μM) in the absence or presence of silibinin or α-tocopherol (10 μM) and incubated for (**A**) 4 and (**B**) 8 h. Caspase-3 activity was measured by a fluorescence assay. Values are expressed as percentage of control caspase-3 activity arbitrarily set as 100%. Bars represent the mean ± SEM of three experiments. *P < 0.01 vs control.

4.5 Study of the oxaliplatin-dependent apoptotic processes in primary rat astrocytes in comparison to the human adenocarcinoma cell line HT-29

4.5.1 Primary rat astrocytes

Aimed to investigate the regulation of the apoptotic processes by oxaliplatin in astrocyte cultures, we studied mitochondria. Eight hour treatment with 100 μM oxaliplatin induced a significant alteration of mithocondrial functionality and the activation of the intrinsic apoptotic pathway. Fig. 16 (left panels) shows that in astrocyte cultures the cytosolic release of cytochrome C from mithocondria was increased 2.5 folds after the anticancer treatment. Moreover 100 μM oxaliplatin induced a significant $O_2^{\cdot -}$ increase, up to 10 times as evaluated by the cytochrome C assay (Fig. 17A). Both silibinin and α-tocopherol strongly reduced the $O_2^{\cdot -}$ levels (Fig. 17A). The anticancer agent disrupted the cellular oxidative equilibrium although it was not a direct oxidant molecule (data not shown). The expression levels of the antiapoptotic protein Bcl-2 was reduced at about 70% by the antineoplastic agent, confirming the activation of the intrinsic apoptotic process (Fig. 18, left bars). The parameters evaluated for the extrinsic apoptotic process were the activity of caspase-8, the expressions of Bid protein and DR5 receptor. These parameters were not significantly altered by oxaliplatin treatment.


Astrocytes
HT-29
control
control
Oxaliplatin 100 µM
Oxaliplatin 100 µM

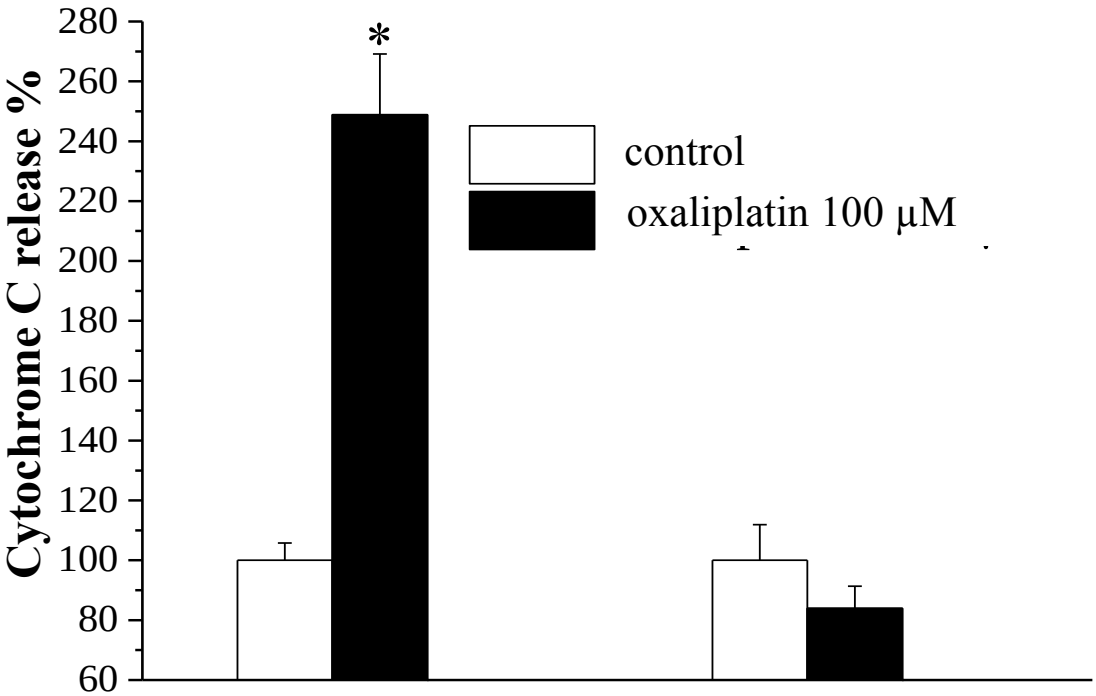

Cytochrome C release %
280
260
240
220
200
180
160
140
120
100
80
60
*
control
oxaliplatin 100 µM

Figure 16. Mitochondrial release of cytochrome C. Astrocytes (10^5 cells/glass slide, left side) and HT-29 (10^5 cells/glass slide, right side) were plated on D-polylisinated glass slides and treated with oxaliplatin (100 μM) for 8 h. Cytochrome C release was measured by immunocytochemistry. Rapresentative images of the quantification are shown above. Values are expressed as percentage of control caspase-3 activity arbitrarily set as 100%. Bars represent the mean ± SEM of three experiments. *P < 0.01 vs control.

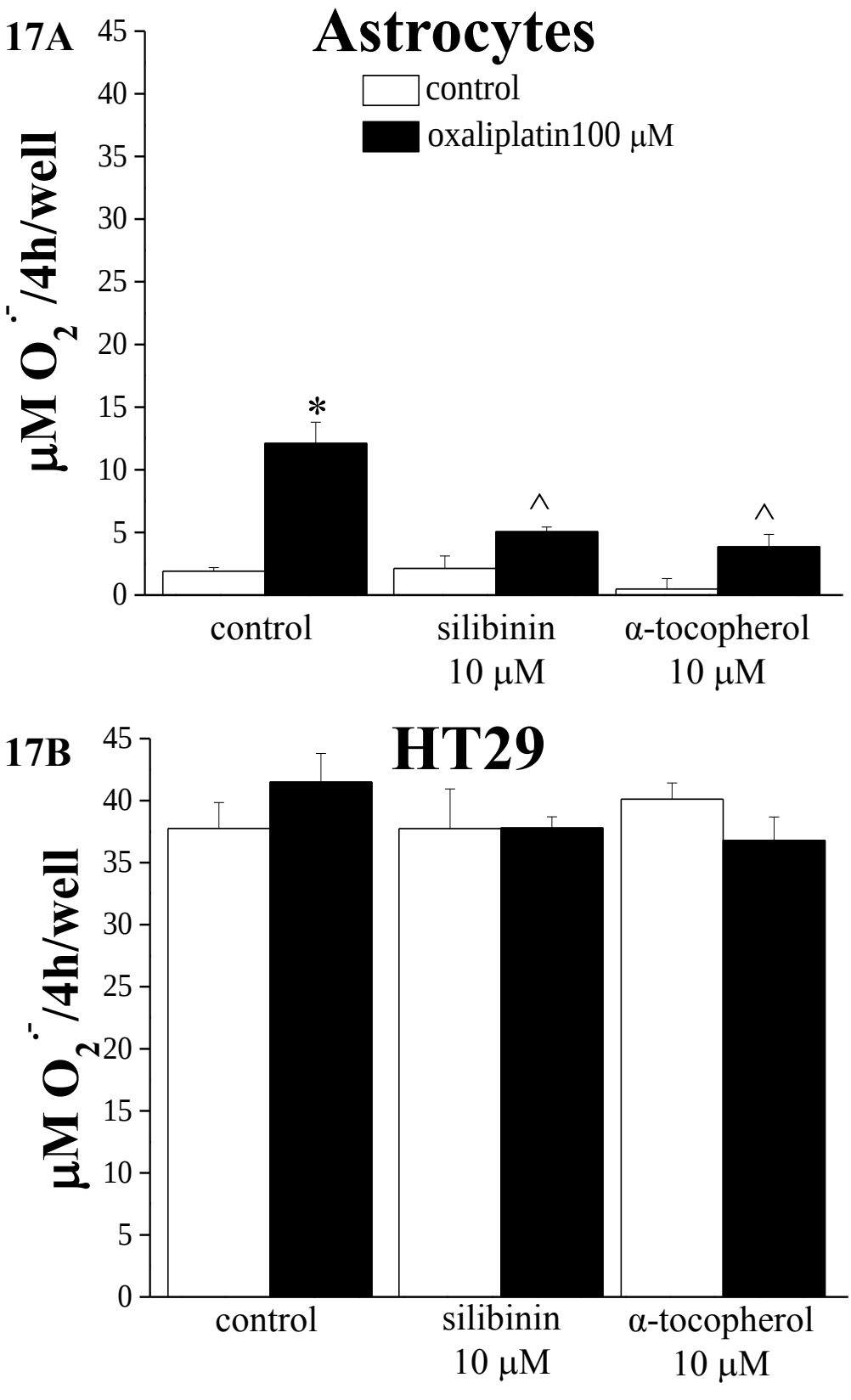


Figure 17. SOD-inhibitable $O_2^{\cdot -}$ concentrations in astrocyte cells (**A**) and HT-29 cells (**B**). Astrocytes ($5 \cdot 10^5$ cells/well) and HT-29 cells ($5 \cdot 10^5$ cells/well) were exposed to 100 μM oxaliplatin for 4 h in the absence or presence of silibinin or α-tocopherol (10 μM). The nonspecific absorbance was measured in the presence of SOD (300 mU/ml) and subtracted from the total value. Values are expressed as the mean ± SEM of three experiments. *P < 0.01 vs control and ^P < 0.01 vs oxaliplatin treatment.

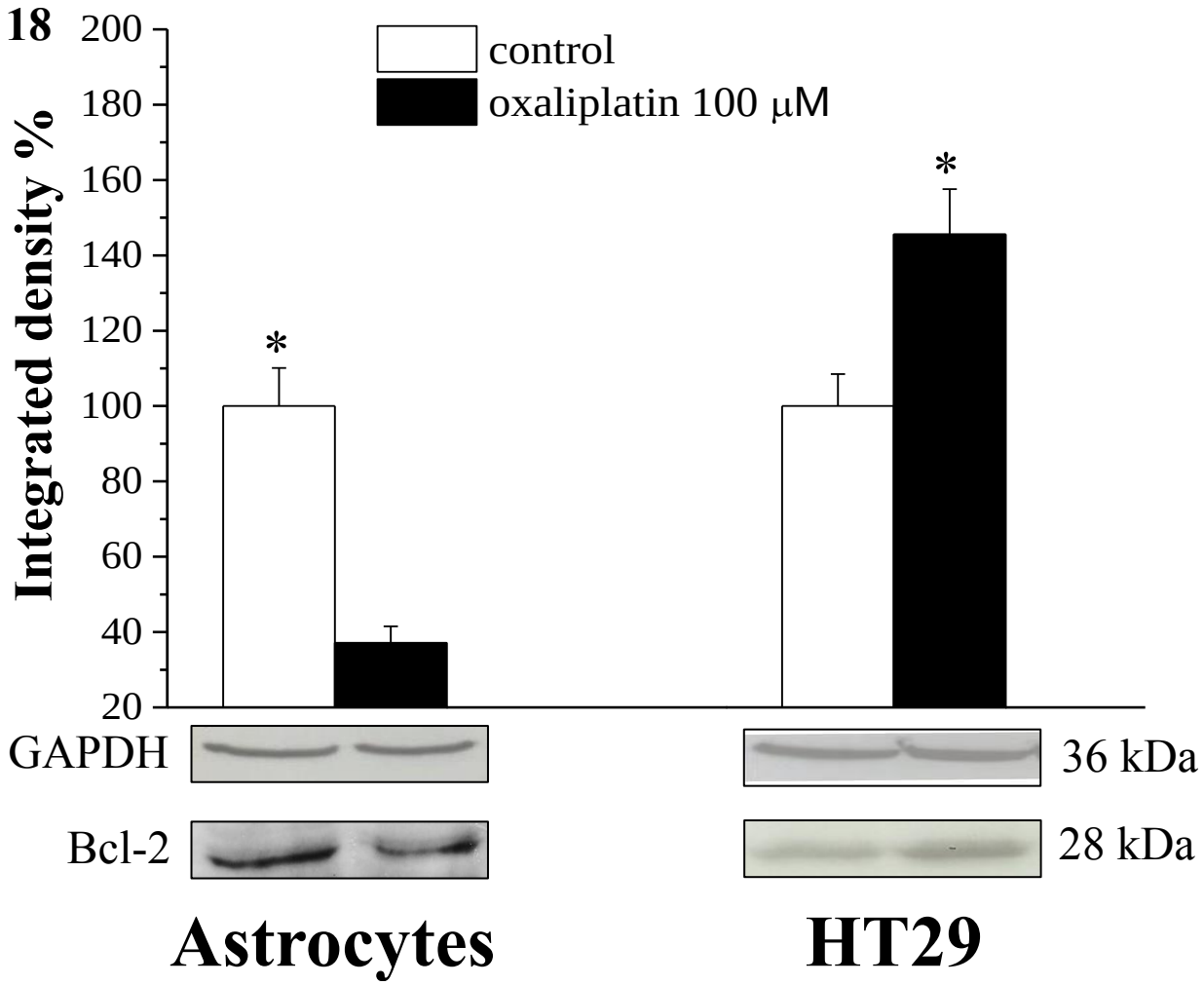


Figure 18. Bcl-2 expression levels in astrocyte cells (left side) and HT-29 cells (right side). Astrocytes (10^6 cells/flask) and HT-29 cells (10^6 cells/flask) were exposed to 100 μM oxaliplatin for 8 h. Western blot analysis was performed on cell homogenates, using a specific antibody. Densitometric analysis (top) and representative immunoblot

(bottom) are shown. Data are expressed as percentage of control. GAPDH normalization was performed for each sample. *P < 0.01 vs control.

4.5.2 HT-29 cells

Differently from astrocyte cells the characterization of the apoptotic phenomenon promoted by oxaliplatin in HT-29 cells, underlined the activation of the extrinsic apoptotic process. After 8 h incubation 100 μM oxaliplatin increased by 1.5 fold the activity of the enzyme caspase-8, central effector of the extrinsic process (Fig. 19, right bars). The expression of Bid, protein able to transfer the apoptotic information from the extrinsic to the intrinsic process, was increased by about 1.7 fold, after 8h treatment with 100 μM oxaliplatin, consolidating the hypothesis of the extrinsic apoptosis activation (Fig. 20, right bars). The anticancer agent did not alter $O_2^{\cdot -}$ levels, but the basal levels were 4 fold higher than those measured in astrocytes (Fig. 17B). This effect was confirmed in another human adenocarcinoma cell line, LoVo cells (data not shown). In the HT-29 cells, differently from astrocyte cultures, the expression level of the antiapoptotic protein Bcl-2 was increased by about 50 % (Fig. 18, right side)

.

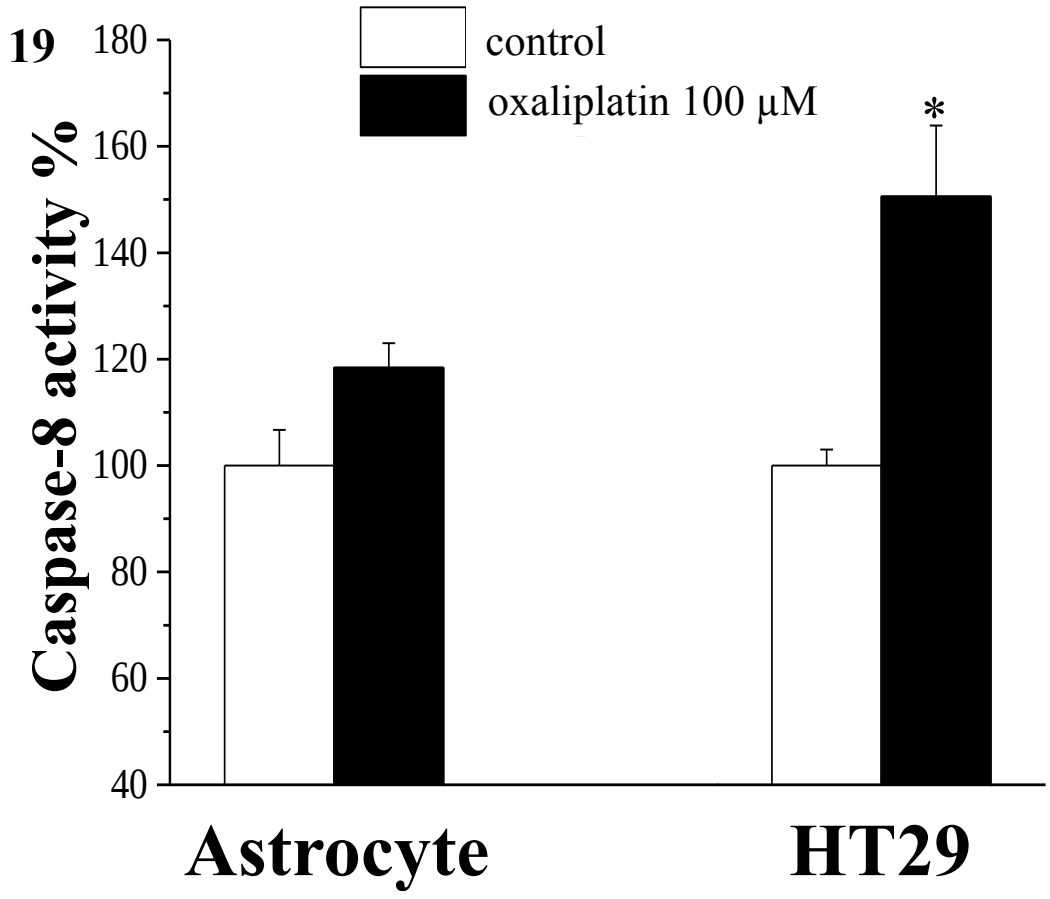


Figure 19. Caspase-8 activity in astrocyte cells and HT-29 cells. Astrocytes ($5 \cdot 10^5$ cells/well) and HT-29 cells ($5 \cdot 10^5$ cells/well) were exposed to 100 µM oxaliplatin for 8 h. Caspase-8 activity was measured with a fluorescence assay. Values are expressed as percentage of control caspase-8 activity arbitrarily set as 100%. Bars represent the mean ± SEM of three experiments. *P < 0.01 vs control.

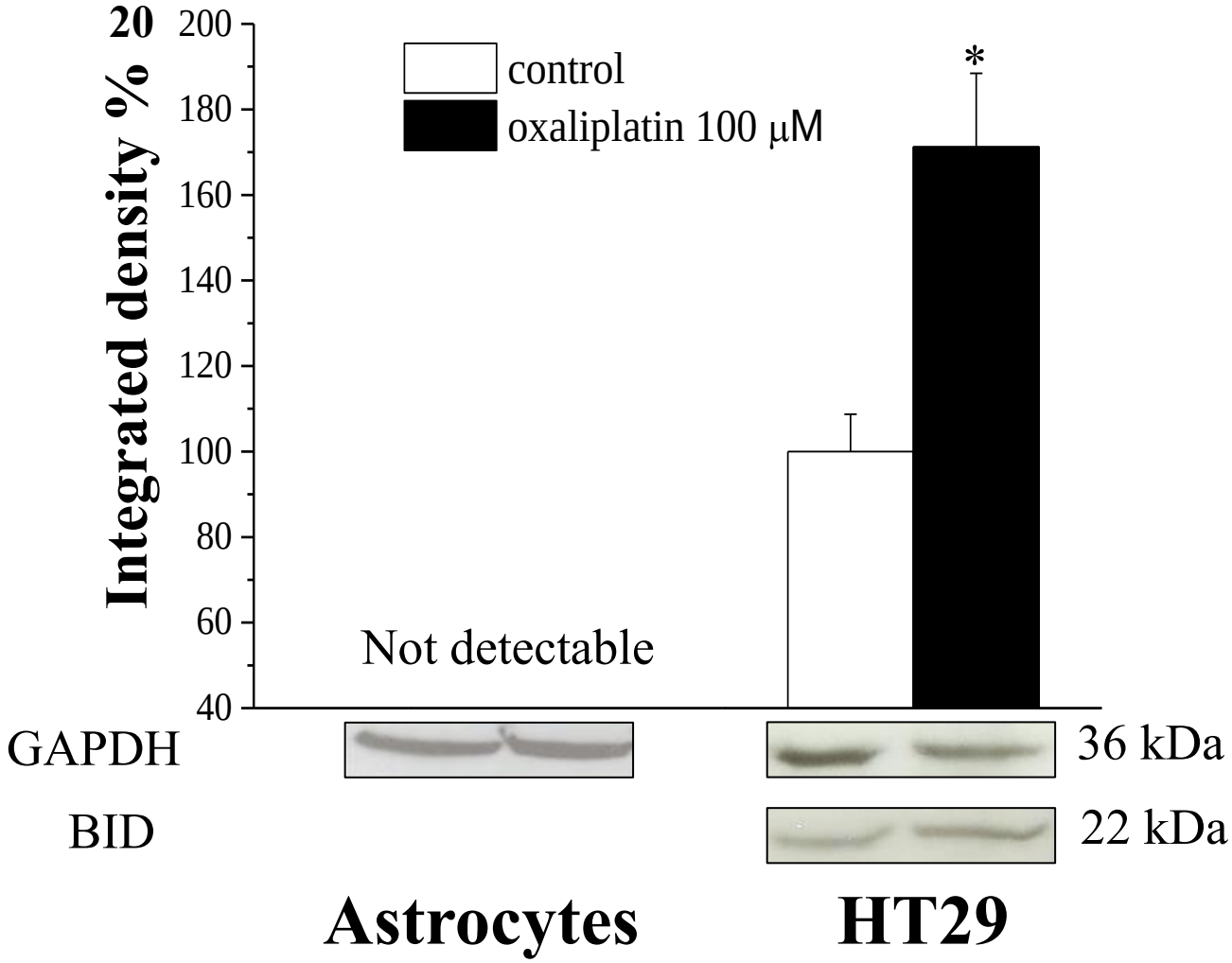


Figure 20. Bid expression levels in astrocyte cells (left side) and HT-29 cells (right side). Astrocytes (10^6 cells/flask) and HT-29 cells (10^6 cells/flask) were exposed to 100 μM oxaliplatin for 8 h. Western blot analysis was performed on cell homogenates, using a specific antibody. Densitometric analysis (top) and representative immunoblot (bottom) are shown. Data are expressed as percentage of control. GAPDH normalization was performed for each sample. *P < 0.01 vs control.

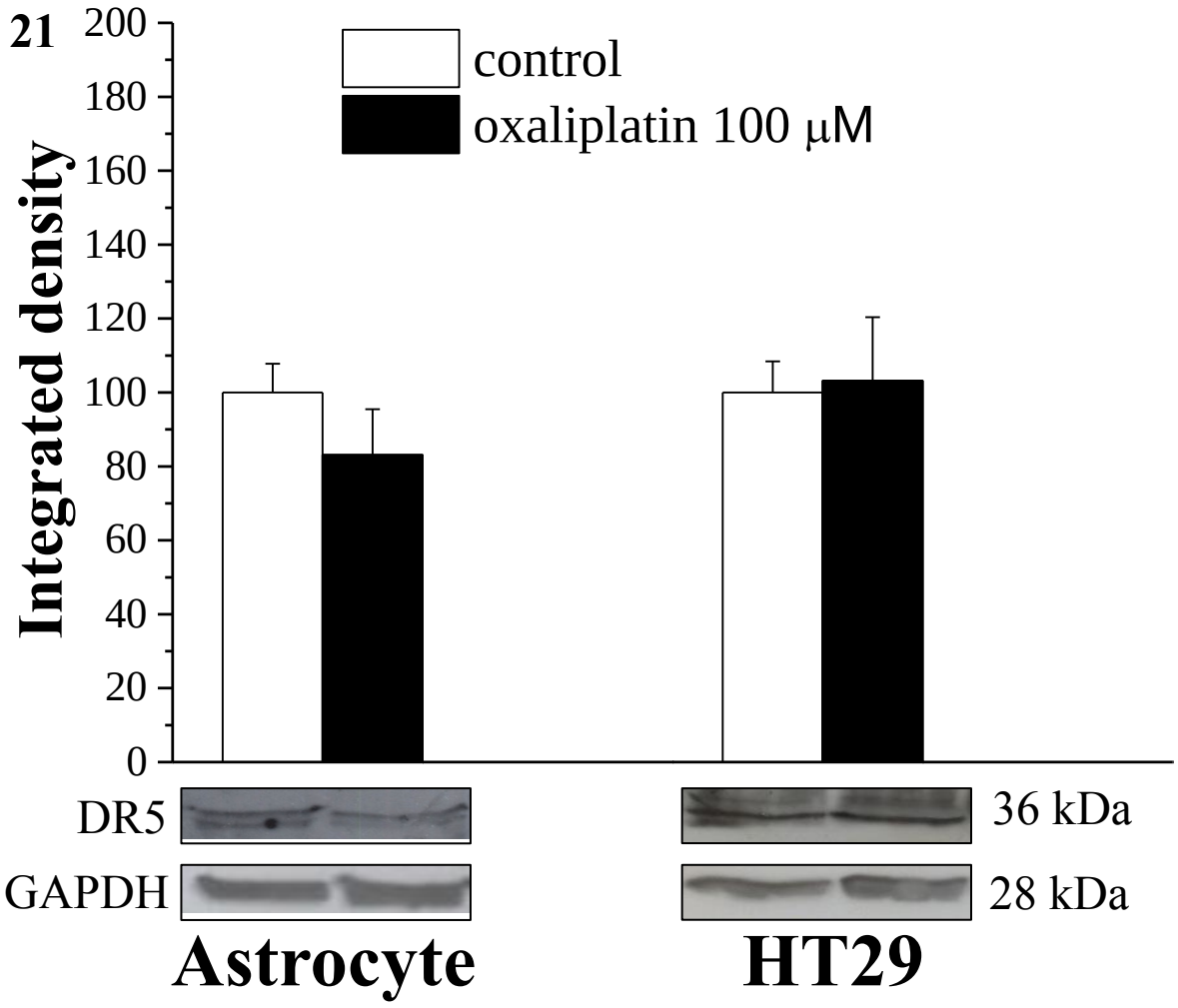


Figure 21. DR5 receptor expression levels in astrocyte cells (left side) and HT-29 cells (right side). Astrocytes (10^6 cells/flask) and HT-29 cells (10^6 cells/flask) were exposed to 100 μM oxaliplatin for 8 h. Western blot analysis was performed on cell homogenates, using a specific antibody. Densitometric analysis (top) and representative immunoblot (bottom) are shown. Data are expressed as percentage of control. GAPDH normalization was performed for each sample.

4.6 Oxaliplatin specific modulation of the apoptotic processes: data on a rat pheochromocytoma cell lines (PC12 cells)

Since astrocyte culture and HT-29 cell line derived from different species, rat and human respectively, to avoid a being-dependent effect, we repeated the measurements on the extrinsic specific marker caspase-8 in the rat tumoral cell line PC12 (pheocromocytoma derived).

Data highlighted that also in PC12 cells oxaliplatin activates the extrinsic apoptotic process. Caspase-8 activity was doubled (Fig. 22) after 8h incubation.

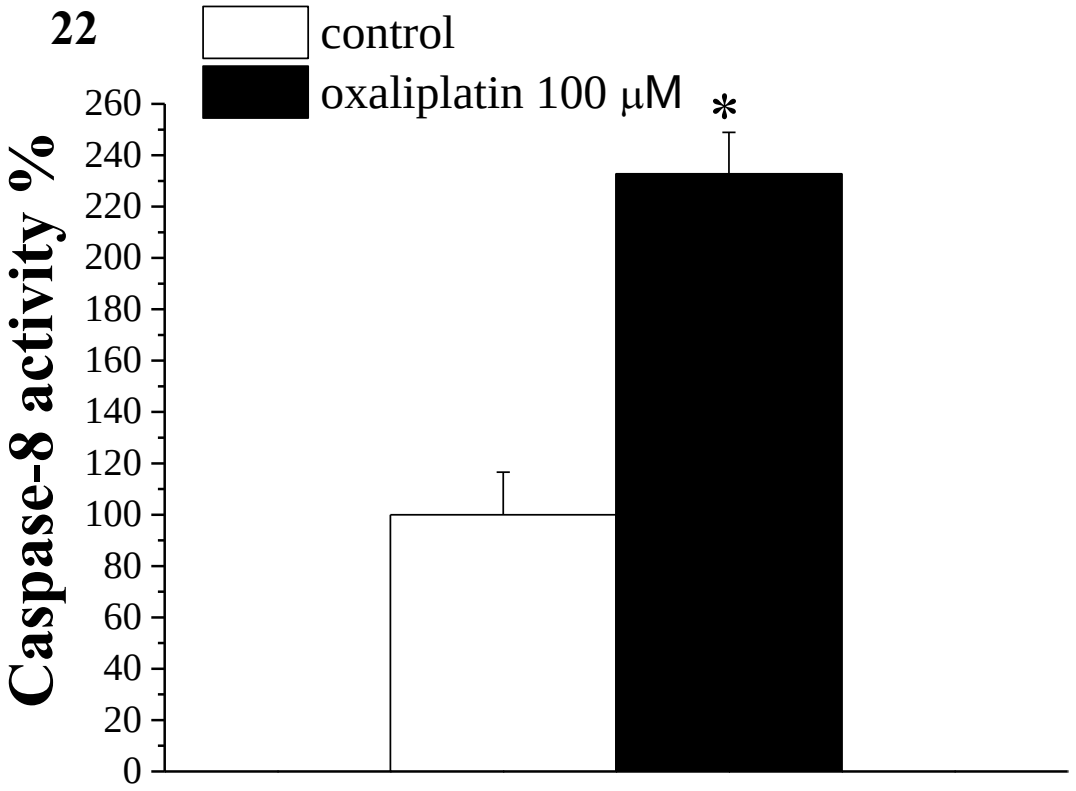


Figure 22. Caspase-8 activity in PC12 cells. Cells ($5 \cdot 10^5$ cells/well) were exposed to 100 μM oxaliplatin for 8 h. Caspase-8 activity was measured by a fluorescence assay. Values are expressed as percentage of control caspase-3 activity arbitrarily set as 100%. Bars represent the mean ± SEM of three experiments. *P < 0.01 vs control.

5.Discussion

The clinical treatment of chemotherapy-induced neuropathy is based on symptomatic drugs. However, in addition to the possibility of side effects, their effectiveness in the treatment of painful neuropathy is not yet definitely proven (Albers et al., 2011). Therefore, the development of active disease-modifying agents is the goal of the research in this field. This issue is hampered by the insufficient knowledge of damage mechanisms.

The present data show that in the *in vivo* model of oxaliplatin-induced neuropathy, oxidative stress is observed at lipid, protein, and DNA levels both in plasma as well as in the nervous system. Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and it is used as an indicator of oxidative stress. TBARS are naturally occurring products of lipid peroxidation that are increased by oxidative stress (Pan et al., 2009). Oxidative modification of proteins by ROS and other high reactive molecules such as hydroxynonenal occurs in physiologic and pathologic processes. As a consequence of the modification, carbonyl groups are introduced into protein side chains by a site-specific mechanism (Disatnik et al., 2000), inducing decreased functionality. Finally, at the DNA level, hydroxylation of guanosine occurs in response to both normal metabolic processes and a variety of environmental factors. Increased levels of 8-OH-dG are associated with the aging process as well as with a number of pathological conditions including cancer, diabetes, and hypertension (Leinonen et al., 1997; Shen et al., 2007).

Oxaliplatin neurotoxicity is described to be localized at dorsal root ganglia level (Ta et al., 2006). As reported by Jacobs et al. (2005 and 2010) after a single administration by i.v. injection (5 $mgkg^{-1}$ i.v.) in nonhuman primates, the oxaliplatin concentration in CSF is limited. Nevertheless, the present results highlight the oxidative damage existing in the spinal cord. On the other hand, data about the oxaliplatin concentration in the central nervous system after repeated administration are lacking.

A significant decrease of the oxaliplatin-induced oxidative state is shown in plasma and in the nervous system of rats repetitively treated with silibilin and α-tocopherol at antioxidant dosage (100 mg kg^{-1}; Muriel et al., 2005; Tomè et al., 2010). The protective effect is concomitant with a decrease of both hyperalgesia and allodynia. On the contrary, acute administration of both molecules are ineffective. These results

exclude a symptomatic analgesic effect of antioxidant compounds leading us to hypothesize a relationship between the activity against oxidative stress and the anti-hyperalgesic properties of both molecules. Clinically used antioxidant agents partially reduce oxaliplatin-induced toxicity without a precise explanation for their limited, beneficial effects (Penz et al., 2001; Lin et al., 2006). Their therapeutic limited effectiveness could be due to the scarce activity of the presently available substances or to the complexity of oxaliplatin neurotoxicity.

Although the oxidative stress promoted by oxaliplatin could be considered only an epiphenomenon, the present *in vivo* data and other *in vitro* evidences (Park et al., 2000; Nassini et al., 2011) suggest a relationship between platinum-induced neurotoxicity and oxidative stress. In this view, focusing on the oxidative damage oxaliplatin-mediated, we set up an in vitro model to deep inside oxaliplatin neurotoxicity and to screen molecules able to reduce it.

In SHSY-5Y cultures oxaliplatin is able to induce lipid, protein, and DNA oxidative damage. Moreover, it causes a concentration-dependent decrease in cell viability. Similar detrimental effects and an increase of $O_2^{\cdot -}$ production are induced by oxaliplatin on isolated astrocytes. However, the oxaliplatin LC_{50} in astrocytes is significantly higher than in SH-SY5Y cells. This distinctive sensitivity to oxaliplatin of the various nervous system-derived cell types may arise from specific cellular characteristics. It should be considered that SH-SY5Y derive from neuroblastoma cells and, although possessing several characteristics of neuronal cells, they conserve some tumoral features (Sharma et al., 1999; Mantha et al., 2012).

However, although astrocytes are generally less susceptible to oxidative injury than neurons, oxidative stress can alter their functions (Choi et al., 2007). Under physiological conditions, glial cells exert neuroprotective effects by providing neurons with substrates for oxidation (Milligan and Watkins 2009) but during neuropathy, dysfunctional glial cells no longer maintain homeostasis and contribute to nervous circuit alterations (Gwak et al., 2012). Nevertheless, silibinin and α-tocopherol decrease the oxidative damages observed in both cell cultures. These results are in line with the antioxidant and protective activities of both molecules in *in vivo* experiments (Di Cesare Mannelli et al., 2012). The parallel between antioxidant protective effects in cellular systems and antineuropathic efficacy in the rat suggests the measure of

cellular oxidative stress as a rapid and valid method for studying features of oxaliplatin neurotoxicity and screening new antioxidant compounds able to reduce oxaliplatin-induced neuropathy. Since that astrocytes show a reactive status at the beginning of pain chronicization in the rat model of oxaliplatin-induced neuropathy (Di Cesare Manelli et al., 2013b) and their dysfunctions in the CNS play a pivotal role in the development and maintenance of chronic pain (Gao and Ji 2010), astrocyte cultured treated with the platin drug can be considered a good model of oxaliplatin neurotoxicity.

The detrimental oxidative effects described in nervous system derived cells in this research were obtained at concentrations ranging from 10 to 100 μM. Although these concentrations are higher than those measured in human plasma during therapeutic infusion at standard dosage (85 mg/m^2; Shord et al., 2002), to our knowledge, the oxaliplatin concentration in the central nervous system has not yet been measured in a human being. Jacobs and co-workers in nonhuman primates (Jacobs et al., 2010) report that oxaliplatin concentrations in the brain extracellular fluid range from 2 to 9 μM/h (AUC 0–4 h) after a single 21–25 mg kg^{-1} intravenous drug infusion over 2 h. According to pharmacokinetic experiments (Shord et al., 2002) oxaliplatin possesses a wide volume of distribution (i.e., 1.5 L kg^{-1}) and a triphasic clearance, which is reflected by its half-life (391 h). Moreover, when oxaliplatin is administered for 4 days consecutively, patients' plasma concentration during the fourth cycle is significantly higher than during cycle 1, suggesting an accumulation of oxaliplatin in repeated treatments (Merkel et al., 2003). Therefore, the concentrations of oxaliplatin used in our experiments might be comparable to the clinical situation in which chronic neuropathy affects approximately 50% of the patients receiving cumulative doses higher than 1000 mg/m^2 (de Gramont et al., 2000).

The increased levels of superoxide anion, induced by oxaliplatin in astrocyte cultures, does not seem to correlate with a direct oxidative property of the molecule because oxaliplatin is not able to induce oxidation in nervous tissue homogenate, when cell integrity is disrupted. Oxaliplatin-induced oxidative stress seems to be related to a cell-mediated effect. One of the main source of free radicals, like superoxide anion (Muller et al., 2004), are mithocondria. Their antioxidant defences work finely to maintain the cellular oxidative state (Fisher et al., 2011). These organelles are related

to neurodegenerative oxidative phenomena (Galea et al., 2012) and mitochondrial alteration has been suggested as a mechanism of oxaliplatin-mediated oxidation (Zheng et al., 2011; Ferrari et al., 2011). Our data demonstrate that in astrocyte cultures, oxaliplatin promotes the release of cytochrome C, index of mithocondria dysfunction and activation of intrinsic (mithocondrial) apoptosis activation. In this primary cultures the activation of intrinsic apoptosis pathway could be due to the reduction of Bcl-2 expression, antiapototic protein that regulated the cytosolic release of cytochrome C (Mignotte and Vayssiere 1998). Moreover, according to Harrison et al. (2007), the cytosolic release of cytochrome is not promoted by Bid, since that its expression is very low in astrocyte cells. The extent of the mitochondrial damage induced by oxaliplatin is able to promote the activation of caspase-3, a hallmark of apoptotic cell death and end effector of both intrinsic and extrinsic apoptosis pathways (Nicholson and Thornberry 2003). Silibinin and α-tocopherol exhibit antiapoptotic properties, limiting the enzymatic activation. Joseph and Levine (2004) demonstrated that apoptotic phenomena are involved in the neuropathic pain-related behavior, as well as the inhibition of caspases 1, 2, 3, 8, and 9 reduces hyperalgesia in rat models of neuropathy induced by anticancer or antiviral chemotherapy. On the other hand Acetyl-L-Carnitine, a neuroprotective agent able to treat neuropathic pain, shows antiapoptotic properties in the rat chronic constriction injury neuropathy model (Di Cesare Mannelli et al., 2007). According to our data, silibinin and α-tocopherol can ameliorate the symptoms of oxaliplatin-induced neurotoxicity reducing oxidative macromolecule alterations and completely preventing apoptosis in nervous cells.

In the cancer cell line HT-29, oxaliplatin incubation induces a concentration-dependent cell mortality and, similarly to astrocyte cultures, a significant increase of caspase-3 activation. Silibinin and α-tocopherol do not influence the oxaliplatin-dependent HT-29 cell mortality. Differently from astrocytes, the antiapoptotic properties of both antioxidants are lost in the tumoral cell line HT-29, in which neither silibinin nor α-tocopherol altered oxaliplatin-induced caspase 3 activation. Moreover, after 4 h incubation these natural compounds exert a *di per se* apoptotic activity, increasing the levels of cleaved caspase 3 substrate in tumor cells. These data, according to the chemopreventive effects described both *in vitro* (Cheung et al., 2007) and *in vivo* (Singh and Agarwal 2006) models, suggest that both molecules might be

used as clinical supportive care in chemotherapy without interfering with the antitumor efficacy of oxaliplatin.

The modulation of caspase-3 activity, exhibited by silibinin and α-tocopherol in astrocytes as respect to HT-29 cells, reflects a different regulation of apoptosis in normal cells vs tumoral cells after oxaliplatin treatment. In HT-29 cells, oxaliplatin selectively promotes the extrinsic (non mithocondrial) apoptosis pathway. Caspase-8 is activated and the expression levels of Bid, a protein able to transfer the apoptotic information from the extrinsic to the intrinsic apoptotic pathway, are increased (Luo et al., 1998).Caspase-8 activation is confirmed also in a rat pheochromocitoma cell line (PC12) suggesting that the modulation of the extrinsic pathway, promoted by oxaliplatin, is specific.

On the contrary, in the tumoral cells, the mithocondrial-regulated apoptosis is unaffected by oxaliplatin, probably by a Bcl-2 increase that expression limits the cytosolic release of cytochrome C. Unlike astrocyte cultures, the superoxide anion levels are not altered by the chemiotherapic agent, although in the human adenocarcinoma colo-rectal cell lines (HT-29 and LoVo) the free radicals concentrations are higher than those measured in astrocyte cultures. This could be explained considering that in cancer cells metabolic processes are faster than those of normal cells. In particular mithocondria do not exert a pivotal role in the cellular metabolic processes as in normal cells (Kim and Dang 2006). Tumoral cells can bypass the organelles activity producing energy by a high rate of glycolysis followed by lattic acid fermentation in the cytosol. On the contrary, the normal cells show a comparatively low rate of glycolysis followed by oxidation of pyruvate in mithocondria (Kim and Dang 2006). Since glycolysis provides most of the building blocks required for cell proliferation, cancer cells (and normal proliferating cells) need to activate glycolysis, despite the presence of oxygen, to proliferate (Lopez-Lazaro 2008). In this view our data suggest that mithocondria functionality of normal cells could be more sensitive against oxaliplatin toxicity than tumoral one.

Taken together our data underline that oxaliplatin-dependent apoptosis is mediated preferentially by the intrinsic apoptosis pathway in nervous normal cells. The extrinsic pathway based on caspase-8 activation seems to be responsible for apoptosis in tumoral cells. Moreover our research highlighted that new antineuropathic agents

should have neuroprotective properties based on mitochondrial preservation and they should not interfere with the activation of caspase-8 promoted by oxaliplatin in tumoral cells.

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