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Chapter 2

Genotoxicity Induced by Ocupational Exposure to Pesticides

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Additional information is available at the end of the chapter

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1. Introduction

Pesticides are used to repel, kill or control certain forms of pests, e.g. animals or plants. These chemical compounds can be divided into three main classes: insecticides, which are used to control insects; herbicides, which are used to destroy unwanted vegetation; and fungicides, which are used to control fungi and their spores, preventing them from damaging plants (Maroni et al., 2000). Pesticides are employed extensively around the world and in recent years their use has even increased. On one hand, extensive use of pesticides in farming has lead to a higher production of pests that damage crops and, on the other hand, pesticide-resistant pests have emerged. Increased crop production demands increased use of pesticides (Mostafalou and Abdollahi, 2013).

The widespread use of agricultural chemicals in the food production and public health sectors has released large amounts of potentially toxic substances into the environment, most of which are unspecific and therefore potentially also target the human organism (Bolognesi, 2003; Dyk and Pletschke, 2011). Humans are exposed to the ubiquitous pesticides, e.g. in form of food contaminations through the production line, but also in the household, workplace, hospitals and schools (Bolognesi, 2003; Aprea et al., 2012).

Exposure to pesticides can induce two kinds of toxic effects: acute and chronic. The acute effects are immediate and include headache, nausea and/or other more serious effects, even death. Chronic health effects occur, when individuals are exposed continuously or repeatedly to foreign substances. In the scientific literature, the effects of acute exposure are more clearly described. In contrast to that, effects of chronic exposure still need to be further investigated,

especially how they are triggered (Ray and Richards, 2001; Sanborn et al., 2007; Kortenkamp et al., 2007).

The degree of danger associated with chemical exposure can be evaluated by health risk assessments. Chemical exposure can be evaluated with respect to a single compound or to complex mixtures. Mixtures of toxins may influence and even amplify the toxicity of individual components through synergies, potentiation, antagonism, inhibition or additive effects (Muntaz, 1995; Reffstrup et al., 2010). The assessment of chronic exposure to mixtures of pesticides should improve the understanding of underlying intoxication mechanisms (Bond and Medinsky, 1995; Sanborn et al., 2007; Reffstrup et al., 2010). Indeed, the number of studies involving chronic exposure to pesticides and their consequences to human health (Muntaz, 1995; Mostafalou and Abdollahi, 2013) in the scientific literature is increasing. Individuals, who are in direct contact with and exposed repeatedly to low levels of pesticides (e.g. agrochemicals) as part of their work (e.g. agricultural or cargo workers, etc.) may therefore provide a good opportunity to study the deleterious effects of chronic pesticide exposure to human health (Bolognesi, 2003).

2. Occupational exposure to pesticides: Toxicology and absorption pathways

Pesticides can be classified according to their chemical structures: carbamates (CBM), dithiocarbamates (DTC), synthetic pyrethroids, organochlorines (OC), organophosphorous (OP) compounds, thiocarbamates, phenoxyacetates (PHE), quaternary ammonium compounds and coumarins (Maroni et al., 2000). The individual toxicity of these compound classes is expressed by the dose inducing lethality in 50% of the specimens in tests with laboratory animals (LD50). During these LD50 tests, usually mice are exposed to a single given dose (Maroni et al., 2000; Suiter and Scharf, 2012). In practice however, the toxicity of pesticides should not be evaluated on the basis of a single dose, but by the absorption of small doses over a given time period (Bolognesi, 2003; Kortenkamp et al., 2007; Reffstrup et al., 2010). In addition, agricultural workers are usually exposed to a mixture of pesticides (Bolognesi, 2003; Kortenkamp et al., 2007; Aprea, 2012) and fundamental aspects such as type and duration of the exposure can severely affect the toxicodynamics of the pesticides (Gammon et al., 2012).

In laboratory tests, toxicokinetic models are important in order to determine the kinetic parameters of the active components and to understand chemical interactions between pesticides (Bond and Medinsky, 1995). Toxicokinetics refer to the route a xenobiotic takes to get into, through, and out of the body. It can be divided into several processes including absorption, distribution, metabolism, and excretion.

The effects of chronic exposure, which pesticides induce in humans, are highly sensitive to several parameters, e.g. dose, duration, and especially the absorption pathway (Aprea, 2012). In agricultural surroundings occupational exposure mostly involves absorption via dermal and/or respiratory routes (Leoni et al., 2012; Aprea, 2012). This type of exposure occurs predominantly during the period of the application of the toxins, e.g. through spraying

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(Ranjbar et al., 2002). The penetration of the skin itself depends on several factors: type of pesticide, temperature, relative humidity, type of exposed unprotected area of the body (e.g. the back of the hand, wrist, neck, foot, armpit or groin), contact time, and the presence of wounds or skin lesions, which greatly facilitate absorption. Cases of absorption through the gastrointestinal tract are also known, albeit less frequent, because larger pesticide particles tend to be deposited in the upper airways of the respiratory tract (Aprea, 2012).

The knowledge about absorption pathways allows a more apt description of real doses of absorption (together with corresponding toxic effects), rather than a description of the dose, which is considered potentially toxic. For example, Ortiz and Bouchard (2012) demonstrated toxicokinetic effects for the fungicide captan after absorption. Unfortunately, it was impossible to isolate the toxic effects resulting from exposure, because the absorption pathways and toxic doses of this compound in humans are not yet known exactly. Several studies have reported a rapid absorption of organophosphates (OPs) via dermal routes, e.g. through connective and mucous membranes, but gastrointestinal and respiratory absorption routes are also known (Stallones and Beseler, 2002). Gammon et al., (2011) reported minor toxicity for CBMs when absorbed throught he skin, but more serious toxic effects after gastrointestinal incorporation. Pyrethroids are generally unstable under environmental conditions and tend to be rapidly absorbed after degradation through hydrolysis, but don't accumulate in the body (Suiter and Scharf, 2012). In contrast, OCs are relatively stable under comparable conditions and can accumulate when absorbed; Absorption doses can moreover be cumulative, depending on the absorption route (George and Shukla, 2011).

Many of the toxicological effects of pesticides have been demonstrated to be mediated by induced redox signaling. Exposure to a wide variety of pesticides induces oxidative stress, as reflected in the accumulation of reactive oxygen species (ROS), lipid peroxidation and DNA damage. For some pesticides, the mechanisms leading to alterations in the cellular redox homeostasis are partially understood. Pesticides can alter cellular redox equilibria via different mechanisms, including their enzymatic conversion to secondary reactive products (e.g. ROS), depletion of cellular antioxidant defenses and/or impairment of antioxidant enzyme functions (Franco et al., 2009; Limon-Pacheco and Gonsebatt, 2009).

Nutrigenomics and nutrigenetics are recent research areas, which seek to understand the effects of diet and nutrients as genetic response modulators to pesticides. The effects of nutrient deficiencies or imbalances, as well as the toxic concentrations of some dietary compounds have been the subject of nutritional research. About 40 micronutrients are required in an optimal human diet, and their levels may vary depending on age, genetic predisposition, etc. (Ames, 1999; Ames 2001). Most interestingly, the genomic damage caused by moderate micronutrient deficiency is of the same order of magnitude as the levels of damage caused by exposure to high doses of environmental toxins (Kym, 2007; Dangour et al., 2010; Wald et al., 2010). Folates and other B-complex vitamins perform key functions in biological processes pivotal to a healthy constitution. Even moderate folate deficiencies may cause genomic damage in the general population. Folates maintain genome stability by regulating DNA synthesis and repair as well as methylation processes. Deficiencies in folic acid can therefore increase chromosomal instability (Beetstra et al., 2005). A major co-factor in the folate metabolism is vitamin B_{12} and

clinical evidence suggests that the inappropriate intake of vitamin B_{12} may result in damage of the DNA. Moreover, chromosome repair mechanisms may be compromised, when vitamin B_{12} concentrations are too low (Swanson et al., 2001). Age and gender are other factors, which may possibly influence the level of DNA damage. Fenech and Bonassi (2011) showed that the damage to DNA increases with age, probably due to a combination of several factors such as inadequate nutrition, occupational or environmental exposure to genotoxins, and a wider variety of other unhealthy lifestyle factors.

3. Pesticide metabolism

Metabolism is one of the most important factors in the toxic profile of a pesticide. During the first steps of the metabolism, chemical compounds are bio-transformed by phase I enzymes, usually the cytochrome P450 (CYP) system. Phase II conjugating enzyme systems, which are present in the glutathione complex, subsequently transform these reaction products into more soluble and excretable forms (Guengerich and Shimada 1991; Eleršek and Filipič, 2011). These enzymatic reactions are generally beneficial, since they help to eliminate compounds from the body. Sometimes however, these enzymes transform otherwise harmless substances into highly reactive forms – a phenomenon known as "metabolic activation" (Guengerich 2001; Abass et al., 2010).

The metabolism reacts towards these xenobiotics in phase I by generating functional and/or polar groups, with the goal to create a substrate for enzymatic reactions in phase II (Hodgson and Goldstein, 2001; Parkinson, 2001). The CYP system comprises a large family of multigenes, which are important in the metabolic phase I of xeno- and endobiotics. Their reactions occur predominantly in the liver, which is the place of subsequent eliminations (Abass et al., 2010). Beyond the hepatic tissue, CYP multigenes can be found in the lung (Lawton et al., 1990), brain (Bergh and Strobel, 1992), and kidney tissue (Hjelle et al., 1986), as well as in the gastrointestinal tract (Peters and Kremers, 1989), and dermal (Khan et al., 1989) and mucous membranes (Eriksson and Brittebo, 1991). The result of these catalytic reactions depends on the type of pesticide and can range from induction to enzyme inhibition (Patil et al., 2003). The toxicity of OPs and CBMs for example, can be monitored after exposure by measuring the esterase activity (Chambers et al., 2001). The high toxicity of OPs and CBMs is attributed to their ability to mimic esters (natural compounds present in biological organisms), such as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Ray et al., 1998; Chambers et al., 2001). Metabolic phase I reactions take place in the liver, where chemical bonds between phosphorus and carbon atoms are cleaved by alkylations (methyl- or ethylation), resulting in the formation of active enzyme centers (Wild, 1975). Trough these phosphorylation processes in the esterase enzymes (AChE and BChE), complexes are formed between the enzymes and the pesticide (Ray et al., 1998; Kamanyire and Karalliedde, 2004; Gupta, 2006). Moreover, the phosphorylation of hydroxyl groups inactivates the enzymatic activity towards substrates and the esterase enzymes lose both stability and function (Pullman and Valdemoro, 1960; Wild, 1975; Ray et al., 1998; Kamanyire and Karalliedde, 2004; Costa, 2006). These interactions can result in

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the formation of reversible and irreversible complexes, depending on the pesticide and the recovery time of the esterase. OPs tend to form more stable, sometimes irreversible, complexes, whereas CBMs usually form less stable and reversible complexes. The resulting complexes can be depleted by enzymes known as "oximes" (Ray et al., 1998; Kamanyire and Karalliedde, 2004). These metabolic depletion transformations can generate metabolites, which a far more toxic than the original foreign species (Abass et al., 2009; Eleršek and Filipič, 2011). During phase I of the metabolism, OPs and CBMs are involved in oxidation and hydrolysis processes. The oxidation reaction is important for the neurotoxicity of CBMs and OPs, since a desulphurization generates metabolites know as "oxons" through CYP enzymes. Oxons are also known as oxygen analogues of pesticides (Eleršek and Filipič, 2011). Usually, CYPs are relatively specific in the detoxification of chemical compounds, e.g.: diazinon is metabolized by CYP2C19; parathion by CYP3A4 / 5 and CYP2C8; chlorpyrifos by CYP2B6 (Eleršek and Filipič, 2011), or by CYP3A4 and CYP2C9 (Leoni, et al., 2012); atrazine, terbuthylazine, ametryn and terbutryn by CYP1A2 (Lang et al., 1997); endosulfan and carbosulfan by CYP2B6 (Abass et al., 2010).

Other metabolic enzymes, such as paraxonases facilitate hydrolysis reactions. Their function is to eliminate OP/CBM-generated oxons, which is achieved by cleavage of a dialkyl phosphate group. However, through this elimination reaction, highly reactive metabolites (e.g. ROS) can be generated. Eleršek and Filipič (2011) considered these to be genotoxic, since they can interact with DNA molecules. Paraxonases play a protective role against the toxic metabolite oxon, but the potential protection is specific to the type of pesticide and depends on the individual's genotype for the PON gene, which expresses these enzymes. Recent studies on animals, demonstrated an increased expression of PON1 as a result of the promotion, signal transduction and transcription factors on the expression of paraoxomase during the metabolism of OPs. However, there are no known relationships between genotypes, which efficiently detoxify through paraxomases, and/or the activities of AChE and BChE (Costa et al., 2012).

Chemical interactions between xenobiotics may cause saturation of enzymes involved in the metabolism (Bond and Medinsky, 1995). Moreover, evaluations involving low doses during exposure to pesticides may not alter metabolism enzymes, which operate without saturation, and therefore mask a possible effect of intoxication (Bolognesi, 2003; Dyk and Pletschke, 2011). Also, the efficiency of conjugation, a process involved in the glutathione complex during phase II of the metabolism, should be proportional to potential excretion (Eleršek and Filipič, 2011). Accordingly, individual genetic variability, involved in the metabolic transformations of pesticides, can influence the observed pathophysiological effects.

4. Pathophysiology of pesticides

Pyrethroids, OPs, CBMs and OCs represent different classes of insecticides (George and Shukla, 2011). The main effect on human health they share can be attributed to neurotoxicity (Dyk and Pletsche, 2011). Pyrethroids, which lack (type I) or contain a cyano group (type II) in the phenoxybenzyl moiety of their chemical structure (Maroni et al., 2000; Nasuti et al.,

2003), interfere with the opening and closing of sodium channels, extending the time of entry for Na^+ cations into the cell (Narahashi, 1996; Spencer et al., 2001). Type II pyrethroids interfere moreover with the chloride channels, blocking the neurotransmitter glutamate receptor (GABA) in the postsynaptic nerve. As a result, the binding of GABA at the receptor site is inhibited and the influx of Cl^- anions into the nerve cell is suppressed (Manna et al., 2006; Suiter and Scharf, 2012). GABA is the major inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates and the absence of synaptic inhibition leads to a CNS hyperexcitability. The same effect can be observed through the incoporation of OCs, especially as the active ingredient in fipronil (Suiter and Scharf, 2012).

OPs and CBMs are neurotoxic due to their inhibition of cholinesterases (AChE, BChE), which interfere with the function of the neurotransmitter acetylcholine (ACh) and long-term effects can be observed (Maroni et al., 2000; Mansour, 2004). AChE and BChE are responsible for hydrolyzing ACh, which is widely distributed in the nervous system of vertebrates (Ray et al., 1998; Chambers et al., 2001). In order to regenerate cholinergic synapses, ACh must be rapidly hydrolyzed by AChE, producing choline and acetic acid after a neurochemical transmission (Namba and Hiraki, 1971).

The inhibition of AChE, caused by OP and CBM insecticides results in an accumulation of ACh at the cholinergic synapses and neuromuscular junctions, eventually causing various signs and symptoms (Maroni et al., 2000; Suiter and Scharf, 2012), Especially muscarinic and nicotinic sites as well as other areas of the CNS are severely affected. Usually, affected receptors are present on the surface of nerve cells (Ray et al., 1998; Kamanyire and Karalliedde, 2004). Due to the effects of AChE on muscarinic and nicotinic receptors, cardiac responses, such as tachycardia, sinus bradycardia, hypertension, hypotension, changes in heart rate and force of heart muscle contraction can be observed. Saadeh et al. (1997) also observed cyanosis and increased serum levels of creatinine and lactate dehydrogenase after OP poisoning. Cardiovascular symptoms occur most frequently after poisoning with pyrethroids, OPs and OCs. Cardiac sodium channel proteins are responsible for both rapid upstroke of the action potential and rapid propagation of nerve impulses through the heart tissue. Thus, their function is central to the origin of cardiac arrhythmias (Balser, 1999). Studies of ventricular myocytes in cats showed that deltamethrin increased the duration of the action potential. The kinetic changes produced in the cardiac sodium channels were similar to those induced by pyrethroids in the sodium channels of the nerve membranes (De La Cerda et al., 2002).

Neuropathy caused by exposure to pesticides is usually related to chronic poisoning cases, since the neurological damage in patients with acute intoxications can be reversed and controlled with adequate treatment (Ray et al., 1998; Ray and Richards, 2001; Costa, 2006; Jayasinghe and Pathirana, 2012). OPs are retained on the endoplasmic reticulum of the axons, promoting apoptosis and injury of the muscle spindle located in the center of the nervous system (involving the spine, spinal cord and cerebellum). This damage is manifested in symptoms such as lethargy, tingling, numbness and weakness of the hip (Ray et al., 1998). Furthermore, elevated risks of developing Parkinson's disease, psychiatric disorders and depressive memory disorders have been discussed (Calvert et al., 2007).

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In a review, Rahimi and Abdollahi (2007) suggested hyperglycemia as another effect caused by chronic exposure to OP pesticides. OPs are able to alter the mechanisms involved in the glucose metabolism and thus potentially induce diabetes in exposed individuals. The risk of the general population to develop type 2 diabetes from exposure to environmental OP insecticides, especially in the form of residual contaminants of food supplies, has also been investigated by Rezg et al. (2010).

OP poisoning results in repeated stimulation of cholinergic nerves, which stimulate nerve fibers in the postganglionic parasympathetic muscarinic receptors. This can cause symptoms such as nausea, vomiting, abdominal pain, diarrhea, and tenesmus (Simpson and Schuman, 2002). The phenoxyacetic acid moieties of some herbicides have been associated with the development of gastric cancer. A study showed that chronic exposures to herbicides can result in a 70% chance to develop adenocarcinomas (Ekstrom et al., 1999). Xenobiotics are mainly metabolized in the liver and various types of enzymes, such as alanine aminotransferase, aspartate aminotrasferase (Gomes, 1999; Sarhan and Al-Sahhaf, 2011), and gammaglutamil transferase, as well as other amino acids and proteins (Gomes, 1999) may be affected by their presence. Forensic analysis in humans has also shown histopathological changes in the liver, e.g. necrosis, fat accumulation, and modified centrilobular sinusoidal dilatation (Seema and Tirpude, 2008). Studies conducted on rabbits showed that after the absorption of OPs, leukocyte infiltration occurred in the liver parenchyma, alongside cytoplasmic vacuolization, fatty degeneration and the emergence of pyknotic nuclei in the hepatocytes (Sarhan and Al-Sahhaf, 2011).

OPs are also able to inhibit enzymes, which are important for the metabolism of mitochondrial antioxidant defenses. These are in turn pivotal to the process of respiration and the generation of ATP (Kamanyire and Karalliedde, 2004; Shadnia et al., 2007). This way, pesticides can be directly linked to oxidative stress conditions via lipid peroxidation, which is a molecular mechanism involved in apoptosis (Rastogi et al., 2009). The mitochondrial ATP depletion leads to a stimulation of proteolytic enzymes and a subsequent DNA fragmentation, resulting in cellular death (Shadnia et al., 2007). Mutagenic effects could be observed through the frequency of micronucleus tests (MN), which - on average - were found to be increased after the exposure to OPs (Bolognesi, 2011). These results can be related to certain types of cancer such as Non-Hodgkin's Lymphoma and Leukemia (Bonner et al., 2010).

Mancozeb is a fungicide, commonly used for a wide spectrum of crops (especially soy) and contains a substance with important effects on human health: ethylene(bis)dithiocarbamate (EBCD). EBCD is easily metabolized into ethylenethiourea (ETU), which decreases the activity of tumor suppression proteins, thus facilitating tumor growth (George and Shukla, 2011; Paro et al., 2012). ETU has also been linked to congenital malformation and thyroid disorders (George and Shukla, 2011). Lower concentrations of ETU can affect the morphology and function of cells in the ovarian follicles of mammals (Paro et al., 2012). The effects of paraquat (1,1-dimethyl-4,4-chloride bipyridylium), which is a prototypical agricultural herbicide, have been described by Ranjbar et al. (2002). It promotes toxic effects mainly in the liver and kidney. The latter is predominantly affected by an unchanged excretion of paraquat in the urine (O'Leary et al., 2008). This results in increased tissue injury through lipid peroxidation, which

is a secondary effect of the excessive generation of ROS and/or a depletion of the antioxidant defenses (Ranjbar et al., 2002; Samai et al., 2010). Nephrotoxicity processes can also be observed, usually as a result of oxidative stress and/or DNA damage (Samai et al., 2010). A recent study has investigated the effects of OPs on the neoplastic skin cells of rats. The main hypothesis was that the exposure to the herbicide glyphosate, a member of OP family, could lead to increased levels of oxidative stress and result in increased levels of DNA damage (George et al., 2010).

During or after the use of paraquat, triazines, OPs and thiocarbamates, the development of respiratory symptoms was observed among farmers (Hoppin et al., 2002). Paraquat can initiate pulmonary fibrosis through the generation of ROS, whereas glyphosate was associated with the development of chemical pneumonitis (Kirkhorn and Garry, 2000). After exposure to major dosage of OPs and CBMs, signaling in muscarinic receptors was affected and OPs were found in the nerves of post-ganglionic parasympathetic fibers, resulting in respiratory hypersecretion, rhinorrhea, bronchospasm, dyspnea, and cyanosis. These symptoms can evolve progressively and end - due to a complete lack of nerve signals - in apnea and respiratory paralysis (Gaspari and Paydarfar, 2007).

Pesticides interfere with the endocrine system and neurobehavioral development. Moreover, reproduction mechanisms are affected via the endocrine function of steroid hormones, which act as agonists/antagonists in the reproductive system (LeBlanc et al., 1996). The normal reproductive development depends on the interaction of steroid hormones with tissue specific receptors. Xenobiotics may affect the balance between androgen, estrogen and progesterone and hindered interactions between steroids and their receptors may have adverse endocrine effects. These interactions have been examined in studies regarding the exposure to CBMs, e.g. in fungicides. The biosynthesis of imidazole resulted in a deficient production of testosterone hormones (DiMattina et al., 1988), whereas chlordecone and endosulfan increased the testosterone metabolism (Le Blanc et al., 1966). These results contribute to the understanding of the causes of infertility in humans exposed to pesticides (Le Blanc et al., 1996). They also help to explain stillbirths, deformities during embryonic development, as well as congenital malformations caused by OPs (Garry et al., 2002; Maurizio et al., 2008). OCs may affect the function of the thyroid gland, especially regarding the level of thyroxine (T4) production (Le Blanc and Wilson, 1996).

Pesticides can also cause immune alterations, e.g. immunodeficiencies. However, these depend on various environmental factors, which are related to changes of cell functions, and the presence of sub-cellular and/or molecular components in the immune system. The immune response furthermore depends on the interaction between antigens and different cells in the immune system, e.g. lymphocytes or macrophages. Adverse effects can be triggered by direct or indirect immune responses, mainly because some pesticides are more selective than others and may not involve all cell types of the immune system. More specifically, an inhibition of esterases could induce a degranulation of mast cells, thus triggering the release of histamine, which could result in allergic reactions of exposed human individuals. The enzyme phospholipase A2 is involved in the signaling of inflammatory processes, interfering with the humoral and cellular immune system and with T-type lymphocytes (Li et al., 2000; Kamanyire and

Karalliedde, 2004; Li, 2007; Li et al., 2009). It can also produce antibodies, autoantibodies and inhibit natural cell killers such as CD5 and CD26, which promote cytotoxicity (Li, 2007; Li et al., 2009). Most of the pesticide metabolites generate free radicals, which are involved in the generation of oxidative stress conditions. The main mechanisms of the immunotoxicity of pesticides therefore usually involve homeostasis of the pro-oxidant agents and antioxidant defenses.

5. Genotoxic damages of pesticides

Exposure to pesticides has been associated with an increase in the occurrence of non-Hodgkin's lymphoma (Hardell and Eriksson, 1999), multiple myeloma (Khuder and Mutgi, 1997), soft tissue sarcoma (Kogevinas et al., 1995), and lung sarcoma (Blair et al., 1983). Pancreatic, stomach, liver, bladder, and gall bladder cancer have also been reported (Ji et al., 2001; Shukla and Arora, 2001). Moreover, relations to Parkison's disease (Gauthier et al., 2001) and reproductive influences (Arbuckle et al., 2001) have been examined. Several reports are concerned with chromosomal aberrations (CA) (Au et al., 1999; Zeljezic and Garaj-Vrhovac, 2001; Jonnalagadda et al., 2012), sister chromatid exchange (SCE) (Shaham et al., 2001; Zeljezic and Garaj-Vrhovac, 2002), micronuclei (MN) (Falck et al., 1999; Pastor et al., 2003; De Bortolli et al., 2009; Da Silva et al., 2012a; Benedetti et al., 2013) and Comet cells (Grover et al., 2003; Zeljezic and Garaj-Vrhovac, 2001; Da Silva et al., 2012b; Benedetti et al., 2013) as a result of pesticide exposure. In general, significantly increased levels of these biomarkers were found, suggesting severe genotoxic effects of these pesticides.

Various studies have reported significant incidences of cytogenetic damage in agricultural workers, floriculturists, vineyard cultivators, cotton field workers and others (Bolognesi, 2011). Studies involving biomarkers of exposure are usually used in order to assess occupational exposure, i.e. to correlate exposure to chemical reagents with health effects (Aprea, 2012). For this purpose, different biomarkers regarding exposure, effect or susceptibility towards xenobiotics are used to express a specific measure of interaction between a given biological system and a genotoxin (Bolognesi, 2003; Aprea, 2012). The influence of genotypes on the cytogenetic damage is the specific ability of individuals to influence genotoxic biomarkers, i.e. to activate or detoxify substances with respect to their potential to induce mutations, cancer and other diseases (Hagmar et al., 1994; Hagmar et al., 1998). A variety of enzymatic isoforms have been suggested to influence the individual's risk of contracting cancer after exposure to genotoxins (Sulbatos, 1994; Clapper, 2000). Genomic stability has moreover been linked to several dietary micronutrients, nutrient imbalances, dietary deficiencies, as well as excessive exposure to environmental mutagens or carcinogens, all of which can potentially increase genetic damage. As previously discussed, deficiencies of folic acid or other vitamin B cofactors (e.g. B_{12} and B_6) may cause impaired DNA repair.

In view of these diverse and complex findings, the investigation of humans exposed to pesticides constitutes to be a highly important research topic. MN tests and comet assays are accurate and practical analysis tools, complying with most of the criteria used in human bio-

monitoring (Fairbairn et al., 1995; Grover et al., 2003; Moller et al., 2000). In order to assess, if a prolonged exposure to complex mixtures of pesticides could lead to an increase in cytogenetic damage, our group has examined individuals occupationally exposed to agricultural pesticides in Rio Grande do Sul (Brazil), and the public health workers occupationally exposed to agricultural pesticides in Piauí (Brazil). We were also interested in the potentially important effects of gene polymorphisms, which encode proteins involved in the xenobiotic metabolization/detoxification of phase I or II. These should influence the DNA repair pathways, which should allow an evaluation of the genetic predisposition of individuals towards their xenobiotic metabolizing capacity, i.e. the individual susceptibility towards genotoxic effects of pesticides. Therefore, we also investigated the polymorphism of the PON, GSTM1, GSTT1, CYP2E1, OGG1 and XRCC1 genes. Apart from observing the occupational exposure of individuals towards pesticides, we were also interested in the influence of micronutrient intake (vitamin B_{12}, B_6 and folates), as well as the influence of MTHFR C677T polymorphism on the observed DNA damage.

In order to study all of the aforementioned aspects, our group conducted an investigation on vineyard workers, which involved a total number of 173 individuals (Rohr et al., 2011). Of these, 108 were agricultural workers exposed to pesticides and 65 were control individuals. As evident from MN tests, the individuals exposed to pesticides showed a high rate of DNA damage ($P<0.001$; Mann-Whitney U test), relative to the control group. In addition, some of the MN results of the exposure group suggested genetic polymorphisms of PON, GSTM1, GSTT1, and CYP2E1. OGG1 and XRCC1 are examples of important proteins in the base excision repair (BER) pathway (Au et al., 2004; Goode et al., 2002; Hao et al., 2004; Muniz et al., 2008). In another study, we evaluated two BER polymorphisms: OGG1 Ser326Cys: rs1052133 and XRCC1 Arg194Trp: rs1799782 as well as the combined genotypes of these polymorphisms with PON1 Gln192Arg. The modifications of the genotoxic susceptibility as a function of pesticide exposure was measured by MN tests and DNA damage induction in the peripheral leukocytes of the vineyard workers. Our study demonstrated that the polymorphisms in the BER pathway could modulate the susceptibility to DNA damage caused by the pesticides. Since this repair pathway is the major cellular defense against oxidative DNA damage, our results corroborate existing evidence, which suggests an involvement of oxidative damage in the pesticide-induced genotoxic effects. Our study also reinforces the importance of considering combined effects of metabolism and repair-variable genotypes on the individual susceptibility towards DNA damage. It seems feasible to conclude that these two processes act cooperatively in determining the final response to pesticide exposure.

Brazil is a major producer of soybeans, which are planted in several federal states, but especially in Rio Grande do Sul (RS). The increasing agricultural use of the land is hereby concomitant with an increased use of pesticides. Soybean workers in this region are increasingly exposed to a wide variety of herbicides and insecticides (especially OPs). A study originating from our research group investigated a total of 127 individuals, of whom 81 were exposed and 46 were not exposed to pesticides (Benedetti et al., 2013). Both groups consisted of residents from the city of Espumoso (RS-Brazil), whose main economic income relies on soy crops. We evaluated comet assays of the peripheral leukocytes and buccal micronucleus

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cytome assays (BMCyt; micronuclei and nuclear buds) in exfoliated buccal cells. We observed significant increases in DNA damage in the pesticide-exposed group relative to control group. We also found the gene PON1 to express the enzyme paraoxonase, which is believed to be involved in the protection against oxidative stress in the OP metabolism. The metabolizing genes PON1, GSTM1, GSTT1 and GSTP1 were evaluated in order to analyze the influence of individual susceptibility in response to exposure. The genetic polymorphisms obtained from the exposure biomarkers showed no influence of the genotype on the DNA damage in the farmers' cells. The exposure to pesticides increased DNA damage and did not change the evaluated metabolizing genes.

Occupational risks for tobacco farmers involve the exposure to very large amounts of pesticides, which are applied to the crop fields. Contact with the pesticides is normally established via the contact to green leaves during the tobacco harvest and through the additional exposure to nicotine. Nicotine poisoning could also lead to "Green Tobacco Sickness" (GTS), which occurs, when workers absorb nicotine via the skin as they come in contact with the leaves of the mature tobacco plant. GTS is characterized by nausea, vomiting, headache, muscle weakness, and dizziness. Our group examined the occupational risk of tobacco farmers, involving 167 individuals, of whom 111 were exposed, and 56 were not exposed (Alves, 2008; Da Silva et al., 2012a; Da Silva et al., 2012b). Subjects were recruited from Venâncio Aires and Santa Cruz do Sul (RS-Brazil) between July and February in the years 2008-2010. Blood and buccal cells were collected twice during the tobacco crop cycle of every year. Once during the distribution period of the pesticides and again during the harvest period. Blood and buccal cells were also collected from a non-exposed control group (office workers, who were living in the same region as the exposed individuals). Our study evaluated exposure biomarkers indicative of early biological effects and susceptibility. Genotoxicity and mutagenicity in the tobacco farmers were investigated by comet assays and micronucleus tests of buccal cells and binucleated lymphocytes, respectively. In order to detect a potential impact of these chemicals on the farmers, superoxide dismutase (SOD), catalase (CAT) and plasma cholinesterase activities, as well as levels of thiobarbituric acid reactive substances (TBARS) were evaluated. Total contents of chemical elements in the blood were examined by particle-induced X-ray emission (PIXE) and cotinine levels were analyzed in plasma samples. In order to establish a possible correlation between a potential genetic predisposition of the metabolism of xenobiotics / repair of DNA damage and individual susceptibility towards genotoxic effects of pesticides and nicotine, farmers were genotyped for several genes. The evaluation of the DNA damage also considered the following secondary parameters: use of protective measures, time after exposure, age, and gender. As tobacco farmers were exposed to complex mixtures of pesticides during the application period, significantly higher levels of DNA damage were found in the exposed group relative to the control group. For the exposed group, the damage to the DNA was three times higher during the application period and four times higher during the harvest period relative to the control group. However, no significant difference in the activity of serum cholinesterase was observed between exposed and control group. Prior studies, examining pesticide workers, were unable to identify any correlation between chronic exposure to OPs and BChE inhibition. During the exposure period, all individuals showed symptoms related to pesticide poisoning and GTS, e.g. headache, abdominal pain, nausea, and

vomiting. We observed in our study that the serum cotinine levels among the non-smoking section of the exposed individuals during the harvest period were significantly increased, suggesting absorption of nicotine through skin contact with tobacco leaves. Nuclear anomalies in the buccal mucosa cells of exposed tobacco farmers (both during the application and harvest period) showed mixtures of genotoxic and cytotoxic substances. A minor discrepancy concerning the mutagenicity was noticed between the two different periods of the tobacco cycle. During the harvest period, higher MN values were observed in buccal cells, relative to the application period. In addition, effects on the extent of pesticide-induced DNA damage and cell death as a result of the genetic polymorphisms of PON1 and CYP2A6*9 were observed. Binucleated lymphocyte responses to genetic damage were evident from higher MN levels in the exposed group (mainly during the application period) relative to the control group. Workers employed in the production of pesticides and farmers who used pesticides showed a higher risk/level of exposure and hence, were more prone to the potential deleterious health effects of pesticides. Besides, many pesticides, which are commonly used on tobacco crops, contain inorganic elements, including Mg, Al, Cl, Zn, and Br, which are known to cause DNA damage. In our study, absolute inorganic element levels in the blood samples of the exposure group (application period) were found to be increased.

Elevated levels of DNA damage in the exposure group were also observed during the harvest period, presumably via contact with green tobacco leaves and tobacco plants during the various cultivation processes and concomitant dermal nicotine absorption. Nicotine has been implicated in the generation of free radicals in human cells, directly addressing the relationship between ROS induction and observed DNA damage. Thus, synthetic and natural pesticides may induce oxidative stress, and lead to increased generation of free radicals as well as subsequent alterations in antioxidants, free oxygen-based radicals, lipid peroxidation, and the quenching of enzyme systems. In the exposure group (application period), only the antioxidant enzyme SOD showed increased activity, relative to harvest and control groups. In the harvest group, levels of body-defending antioxidant mechanisms (SOD and CAT) were increased, in order to overcome the induction of oxidative stress. These results indicate that the level of lipid peroxidation was significantly different in the harvest group relative to application and control groups. It is feasible to assume, that the internal antioxidant stimulation in the body were insufficient to scavenge all the free radicals and thus compensate for the increased levels of lipid peroxidation. Age and personal protective equipment (PPE) also showed an influence onto the results obtained from the MN tests. An increase of MN levels corresponding to age was observed for both groups (exposed and control). Interestingly, significant differences were observed between exposed individuals (application period) with complete PPE, relative to those without.

The effect of individual genotypes of metabolism genes on the level of the different biomarkers (comet assay and MN tests in binucleated lymphocytes) was examined in the exposed group. Increased damage of GSTM1 in the application group and an increased damage of CYP2A6*1/*1 in the harvest group were observed. The individual genotype of DNA repair genes in the exposure groups did not show any influence on the different biomarkers analyzed in this study. Our study demonstrates once more the importance of occupational training for farm

workers, regarding safe working practices and safe working environments. Developing countries should use such data to establish occupational safety rules when using pesticides (especially in the context of tobacco crops) in order to minimize occupational risks for the workers involved.

We also evaluated the influence of micronutrient intake (vitamins B_{12} and B_6, folates) and MTHFR C677T polymorphism on DNA damage in the exposed individuals (Fernandes, 2012). We examined 110 individuals of both genders (average age: 42.3 ± 13.3 years), living and working in the city of Venâncio Aires (RS-Brazil). The examined exposure time was 30.3 ± 15.6 years. The results showed increased levels of MN in lymphocytes and modified consumption of folates and $B_{12,}$ ($p = 0.030$ and $p = 0.014$, respectively). No significant correlation between DNA damage (MN frequency, comet assay) and age, gender, smoking, years of exposure or BMI could be observed. Similar results were obtained for the genetic polymorphism of *MTHFR C677T*. A diet with appropriate folate and vitamin B_{12} supplements was able to facilitate adequate DNA repair.

Another study originating from our group followed, for over 30 years, a group of public health workers, concerned with endemic diseases. During their work, these individuals have been exposed to considerable amounts of genotoxic and mutagenic pesticides, which are used in vector control programs. Our study with this group therefore aimed at the evaluation of the mutagenicity (MN tests in buccal cells) caused by the occupational exposure to pesticides in the "Território Entre Rios" (Piauí-Brazil) (Fianco, 2013). The study included 129 individuals, of whom 66 were public health workers (exposed group), and 63 individuals without occupational exposure to pesticides (control group). Mutagenic events were manifested through the presence of significant increased numbers of MN (14.7 ± 2.7), binucleated cells (5.9 ± 1.1) and nuclear buds (10.2 ± 1.8) in the exfoliated oral mucosa cells of the workers, relative to the control group (4.2 ± 0.9, 2.8 ± 0.8, and 3.9 ± 0.9, respectively; Mann-Whitney test). However, age, gender, exposure time, smoking, drinking, or diet did not influence the DNA damage parameters examined. According to these results, the occupational exposure of public health workers to pesticides induces mutagenic damage. Even though public health workers should be aware of the risks they are exposed to, the proper use of personal protective equipment could still be improved.

6. Conclusions

Our findings show in general that agricultural workers exhibit higher levels of DNA damage in somatic cells, suggesting that pesticide exposure is a potential health risk for these workers. In addition, it was possible to correlate these results to specific genetic susceptibility, to the absence or inappropriate use of PPE and to dietary habits. It became evident that continuous education is very important for exposed workers, in order to minimize the deleterious effects of the occupational exposure and the risk of contracting work-related diseases. Chronically exposed individuals were more susceptible to the clastogenic effects of pesticides. Significant differences in the cytogenetic damage were detected in individuals with symptoms of chronic

intoxication (Zeljezic and Garaj-Vrhovac, 2001). Furthermore, others studies observing agricultural workers demonstrated an increase in chromosomal damage during the spraying/application season, when pesticides were used intensively (mainly in workers not using PPE). The use of PPE seems to be beneficial for the workers, which is evident from reduced cytogenetic effects (Shaham et al., 2001). The DNA damage (CA, MN and SCE) could be correlated with the exposure duration in many of these investigations (Bolognesi et al., 1993; Joksic et al., 1997; Shaham et al., 2001; Bolognesi et al., 2002; Bolognesi, 2011), and moreover seem the clastogenic effects to be cumulative for a continuous exposure to pesticide mixtures (Bolognesi, 2003; Bolognesi, 2011).

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