BIOHUMORAL TESTS IN CHRONIC PESTICIDES EXPOSURE

PAOLA MARIANGELA FRAZZETTO[1], GIULIA MALAGUARNERA[1], CATERINA GAGLIANO[2], FRANCESCO LUCCA[1], MARIA GIORDANO[1], LIBORIO RAMPELLO[3], LUIGI RAMPELLO[3], MARIANO MALAGUARNERA[1]
[1]Research Centre "The Great Senescence", University of Catania, [2]Institute of Ophthalmology, University of Catania, [3]Department of Neurology, University of Catania

[Test bioumorali in esposizione cronica pesticidi]

ABSTRACT

Pesticides are an important hazard in agriculture. They are used throughout the world and their use is strictly regulated. To assess the effects of exposure to complex mixtures of pesticides, enzyme activities are among the biomarkers that may be used to detect these effects before adverse clinical health effects occur. Exposure to pesticides for a prolonged period affects the normal functioning of different organ systems, such as the liver. Chronic liver fibrosis is the most frequent consequence of pesticides exposure. Various markers are commonly used to facilitate diagnosis and to evaluate treatment efficacy. Significant increase is found for transaminases, LDH, CK, reflecting cytotoxicity. The serological tests of liver fibrosis include the main extracellular-matrix (ECM) constituents, which are broken down by a family of enzymes known as matrix metalloproteinases (MMPs). They are useful to assess the speed of liver fibrogenesis.

Key words: Pesticides, adverse effects, chronic hepatic injury, liver fibrosis, biochemical markers, serological tests.

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Introduction

Pesticides are chemical or biological substances that are used to kill or control harmful living organisms in our environment. They include herbicides, fungicides, insecticides, rodenticides, soil sterilants and wood preservatives, all of which may find use on farms. In addition, some insecticides are formulated as veterinary medicines, such as sheep dips. Pesticides are biologically active and this renders them intrinsically more hazardous than most other classes of industrial products.

Among the many occupational hazards encountered in agriculture, the intensive use of pesticides has significantly contributed to environmental pollution problems.

Accidental exposure or overexposure to pesticides can have serious implications. The improper use of pesticides may engender biological effects beyond those for which they were originally manufactured. Adverse effects may be caused not only by the active ingredients and the associated impurities, but also by solvents, carriers, emulsifiers, and other constituents of the formulated product[1, 2].

Several studies showed an association between health effects and exposure to insecticides[3].

Pesticide-related health problems usually manifest as a series of symptoms depending on severity of exposure. Disorders of the cardiovascular system, nervous system, sensory organs, and respiratory system, and reduced lung function have been reported after exposure to pesticides. Skin disorders, including dermatitis, headache, and nausea, have also been reported[4].

Occupational pesticide exposure has been linked to acute health effects including rashes, skin, eye and respiratory illness, and death[5, 6]. Chronic effects, including neurological and reproductive

effects and cancer, are more difficult to ascertain, but studies have found associations between pesticide exposure and these effects[7, 8].

In particular, mild organophosphate poisoning manifests in the form of malaise, vomiting, nausea, diarrhea, loose stools, sweating, abdominal pain and salivation[9, 10].

Moderate poisoning includes dyspnea, decreased muscular strength, bronchospasm, miosis, muscle fasciculation, tremor, motor incoordination, bradycardia, and hypotension/hypertension. Severe manifestation could result in coma, respiratory paralysis, extreme hyper secretion, cyanosis, dermatitis, asthma exacerbation, liver damage, sensory peripheral nerve defects, chronic neurobehavioral and motor dysfunction.

Chronic hepatic injury is a relatively common disorder caused by pesticides exposure. It is defined pathologically by ongoing hepatic necrosis and inflammation of the liver, often accompanied by fibrosis. Chronic hepatic injury may progress to cirrhosis (15–20% in the case of chronic HCV) and predisposes to HCC[11]. The effects of chronic toxicity, as with acute toxicity, are dose-related. In other words, low-level exposure to chemicals that have the potential to cause long-term effects may not cause immediate injury, but repeated exposures through careless handling or misuse can greatly increase the risk of chronic adverse effects.

The toxicity of a pesticide can be measured in several ways. The estimated doses of pesticides received by pesticide workers represent significant percentages of standard toxicity measures, especially for chronic endpoints.

The potential for chronic illnesses from pesticide exposure is much higher than for acute toxicity for the pesticides examined. Acute toxicity of a pesticide refers to the effects from a single exposure or repeated exposure over a short time, such as an accident during mixing or applying pesticides. It can be measured as acute oral toxicity, acute dermal toxicity or acute inhalation toxicity[12].

Chronic toxicity refers to the effects of long-term or repeated lower level exposures to a toxic substance. The effects of chronic exposure do not appear immediately after first exposure and may take years to produce signs and symptoms.

The chronic poisoning effects include:

• Carcinogenicity, ability to produce cancer or to assist carcinogenic chemicals.

• Mutagenicity, ability to cause genetic changes.

• Teratogenicity, ability to cause birth defects.

• Oncogenicity, ability to induce tumor growth (not necessarily cancers).

• Liver damage, death of liver cells, jaundice (yellowing of the skin), fibrosis and cirrhosis.

• Reproductive disorders, such as reduced sperm count, sterility, and miscarriage.

• Nerve damage, including accumulative effects on cholinesterase depression associated with organophosphate insecticides.

• Allergenic sensitization--development of allergies to pesticides or chemicals used in formulations of pesticides.

Prolonged exposures to pesticide affect multiple organs including liver which can be detected by serum enzymes and other biochemical parameters among farm workers. Main effects of long-term pesticides exposure appear on the liver. Pesticides exposure is one of the environmental factors hypothesized to increase the risk of HCC.

Pesticides are considered to be possible epigenetic carcinogens through one or several mechanisms, such as spontaneous initiation of genetic changes, cytotoxicity with persistent cell proliferation, oxidative stress, inhibition of apoptosis, suppression of intracellular communication and construction of activated receptors. In chronic liver diseases, liver cell death is a prominent feature and correlates with worsening fibrosis. Fibrosis is the consequence of an overactive wound healing process in response to the injury.

Laboratory tests of chronic hepatic damage

Chronic hepatic injury is a common disorder defined pathologically by ongoing hepatic necrosis and inflammation of the liver, often accompanied by fibrosis. Liver fibrosis represents the final common outcome of chronic liver injury and is often progressive, eventually evolving into cirrhosis. Liver fibrosis is a complex process involving production and deposition of insoluble components that constitute the extracellular matrix (ECM).

These components can be divided into collagens (type I and III and less type IV, V and VI), no collagenous glycoproteins (fibronectin, laminin, vitronectin), proteoglycans and a polysaccharide (acid hyaluronic, HA). Increased levels of extracellular matrix components are useful for monitoring evolution of liver fibrosis.

The cell death can occur by one of two mechanisms: necrosis or apoptosis. After the toxic expo-

sure to hepatocytes undergo apoptosis and hepatic stellate cells migrate to the site of injury to engulf the apoptotic bodies. This engulfment promotes activation of the hepatic stellate cells to hepatic myofibroblasts, and in their activated state these cells promote deposition of extracellular matrix and scar formation in the liver[13].

Clinical symptoms and signs of liver disease are often unreliable in assessing disease severity in patients with compensated liver disease. A variety of symptoms including fatigue, itching, and right upper abdominal discomfort, as well as impaired quality of life, occur in patients with chronic hepatitis C, but symptoms and quality of life scores correlate poorly with liver disease severity[14] [15].

Various biochemical, functional or morphological criteria are commonly used as surrogate markers to facilitate diagnosis and to evaluate treatment efficacy. In fact, for hepatotoxicity laboratory, immunological and genetic data have to be considered as surrogate markers, partially so under certain test conditions. Biomarkers used in human health studies are typically divided into three classes: biomarkers of exposure, effect and susceptibility.

Biomarkers of exposure involve measurements of parent compound, metabolites or DNA- or protein adducts and reflect internal doses, the biologically effective dose or target dose. Biomarkers of effects could be changes on a cellular level, such as altered expression of metabolic enzymes but could also include markers for early pathological changes in complex disease developments, such as mutations and pre-neoplastic lesions. Biomarkers of exposure are preferably specific for the chemicals of exposure, while biomarkers of effect are often unspecific for the agent in question.

Solvents may damage liver cells and liver transaminases may be used to monitor liver damage after combined or mixed exposure. In workers exposed to a mixture of solvents, different biochemical parameters of liver function are measured, such as alkaline phosphatase (ALP), total bilirubin (TB) and aspartate aminotransferase (AST) and these markers show significantly higher levels in these workers.

Laboratory tests that are routinely included in the evaluation of patients with fibrosis include a serum panel of liver tests: albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), prothrombin time, and complete blood counts.

Results of serum tests are always compared with histological findings.

The group of routine tests includes liver enzymes: ALT, AST, ALP, level of bilirubin, platelets count, markers of infectious diseases, autoantibodies. Markers of fibrosis including: collagens: N-terminal peptide of type III procollagen (PIIINP), type IV collagen 7s domain(IV-7S); proteoglycans: hyaluronic acid (HA)[16]; glycoproteins: laminin (LN)[17]; human cartilage glycoprotein 39 (YKL-40)[18]; collagenases and their inhibitors: metaloproteinases (MMPs), tissue inhibitor of metalloproteinases (TIMPs)[19]; cytokines: transforming growth factor β1 (TGF-β1), platelet-derived growth factor (PDGF), tumor necrosis factor β (TNF-β).

Serum ALT levels reflect liver injury, but the correlation between ALT levels and necroinflammatory and fibrosis scores is poor[20, 21]. Despite the poor quantitative correlation, most patients with persistently normal ALT levels (at least 3 normal values over a 6 to 12 month period) have less inflammation and fibrosis[22, 23] and slower rate of fibrosis progression compared with patients with elevated ALT levels.

Non-routine markers are components of extracellular matrix, which are overproduced by activated stellate cells. Liver fibrosis is associated with deposition of several proteins in the liver. Among the proteins produced as part of fibrosis are collagen, laminin (LN), elastin, and fibronectin; metalloproteinases and their inhibitors; and enzymes produced in collagen synthesis such as lysyl and prolyl hydroxylase. Various proteoglycans, such as hyaluronate (acid hyaluronic, HA), are also produced in the process of fibrosis. The plasma concentrations of proteoglycans, proteins of fibrosis and their precursors[24, 25], are correlated to the degree of fibrosis.

Hyaluronic acid (HA)

The serum fibrosis indices, including HA and LN, could reflect the activity of liver fibrosis to some extent. Serum Hyaluronic acid (HA) has been one of the most studied mark¬ers of fibrosis. Hyaluronic acid (HA), a glycosaminoglycan distributed in the connective tissue, is a component of the liver extracellular matrix, which is synthesized and degraded in the liver sinusoidal cells. Its concentration in the serum increases as liver fibrosis progresses because of an increase in its synthesis by activated hepatic stellate cells and in later fibrosis stages also because of reduced clearance by the liver sinusoidal endothelial cells[26, 27].

The primary role of testing for serum hyaluronic acid level is to identify patients without cirrhosis (greater than 90% negative predictive value). The high levels of HA observed in patients with chronic liver disease, have been related with a decreased function of the endothelial sinusoidal cells.

Non-routine tests, which reflect fibrosis, are extracellular matrix remodeling markers: amino-terminal propeptide of type III collagen (PIIIP), matrix metalloproteinase (MMP), tissue inhibitor of matrix metalloproteinase (TIMP), type IV collagen (CL-4). These markers are used alone or in combination with serum chemistries. Studies have shown that serum HA has a better ability to predict fibrosis than procollagen type III amino terminal peptide (PIIINP), laminin, TGF-β, and a number of other clinical and biochemical variables.

Amino-terminal propeptide of type III collagen

Amino-terminal propeptide of type III collagen (PIIIP) is a marker of deposition rate of type III collagen. The serum concentration of PIIINP reflects the turnover of type III collagen. The N-terminal propeptide of type III collagen (PIIINP) levels have been most extensively evaluated in patients with chronic hepatic damage. Serum levels of early fibrogenesis and inflammation correlate better with histologic inflammation and are inferior to serum hyaluronic acid levels in predicting hepatic fibrosis.

Index of PIIIP and matrix metalloproteinase (MMP-1) significantly correlates with fibrosis score and is considered better than hyaluronic acid and TIMP-1.

Matrix metalloproteinase(MMP)

Changes of MMP reflect pathologic matrix degradation in liver. The most important enzymes are MMP-2 (also called gelatinase A or 72-kDa type IV collagenase) and MMP-9 (gelatinase B or 92-kDa type IV collagenase), which reflects type IV collagen. The increased expression of MMP-2 is characteristic of cirrhosis.

Tissue inhibitor of matrix metalloproteinase (TIMP)

Significant increases of TIMP 1 and TIMP 2 have been observed in chronic liver disease at any stage of the fibrotic process.

Type IV collagen (CL-4)

CL-4 is a sensitive marker for active fibrosis and the elevation of serum type IV collagen level reflects the enhancement of type IV collagen synthesis and deposition in the liver tissue at the stage of active fibrosis in liver disease. Elevated serum amino-terminal propeptide of type III collagen, prolylhydroxylase (PH), collagen type IV and matrix metalloproteinase-1 were seen in cirrhosis of various cause, laminin and CL-IV in alcoholic hepatitis, hyaluronic acid, tissue inhibitor of metalloproteinase TIMP-1and TIMP-2, in chronic hepatitis.

Serum CL-IV, MMP-2 and TIMP-1 (but not laminin, MMP-1 or MMP-3) were elevated in hereditary hemochromatosis, and only CL-IV and MMP -2 correlated with severity of hepatic fibrosis. Prolylhydroxylase is a marker of collagen synthesis and reflects the grade of fibrosis.

Type VI collagen

Type VI collagen is a minor but essential matrix component in the liver. Type VI collagen gene expression, together with other connective tissue components, including type I collagen, is activated in the early stages of the fibrotic process. Type VI collagen accumulation may contribute to the distorted architecture and functional impairment of the liver in hepatic fibrosis.

Laminin (LN)

Laminin, a major noncollagenous, high molecular mass glycoprotein of basement membranes, increases in fibrotic liver. Increases in laminin concentration are positively correlated with the extent of fibrotic transition of the liver. Discrimination between fibrotic and cirrhotic stages of chronic liver diseases by means of laminin assay is better than the aminoterminal propeptide of type III procollagen. The increase of laminin is relatively independent of the disease etiology.

YKL-40

Recently, serum YKL-40 has been reported to be superior to hyaluronic acid levels in predicting hepatic fibrosis. YKL-40 is a mammalian member of a chitinase family with a molecular weight of 40 kd. The physiologic functions of YKL-40 are unknown. The protein is expressed in human liver and may be involved in the remodeling of the extracellular matrix. A recent study of different causes of chronic liver disease found that serum YKL-40 levels were increased, even in patients with mild fibrosis, and correlated better with histological fibrosis scores than serum hyaluronic acid levels.

Fibronectin

Fibronectin plays a role in liver fibrosis for discriminating patients with liver fibrosis from those with no liver fibrosis.

Cholinesterases

Blood cholinesterases have been used for monitoring exposure to pesticides. The

cholinesterase present in neural tissues, and also in erythrocytes, is known as true acetylcholinesterase (AChE). Cholinesterase actually corresponds to two enzymes: acetyl cholinesterase and butyryl-cholinesterase (also called plasma cholinesterase). The activity of cholinesterase enzymes in the blood can be utilized as a biomarker for the effect of organophosphates. An exposed person will show abnormal low levels of activity of cholinesterase enzymes measured in the serum or in red blood cells (as RBC cholinesterase). RBC cholinesterase is more difficult to measure and it is depressed more slowly than plasma cholinesterase.

Certain pesticides also exhibit preferential inhibition of either enzyme. Furthermore, the serum bile acids are the most sensitive markers for detecting liver injury, suggesting that serum bile acids could be a valuable biomarker of hepatotoxicity caused by organic solvents.

Δ-Aminolevulonic acid dehydratase

Changes in d-aminolevulonic acid dehydratase (ALA-D), an erythrocyte enzyme, have been reported after exposure to different pesticides. This enzyme is a sensitive biomarker that can be used together with AChE for the assessment of long-term health risks of workers exposed to pesticides.

Gamma glutamyl transferase (GGT)

Serum gamma glutamyl transferase (GGT) plays an important role in the metabolism of pesticides. GGT as well as acetylcholinesterase (AChE) activities are found to be altered following intoxications by pesticides. GGT is also considered to be among the oxygen free radical (OFR) enzymatic scavengers that protects against oxidative stress induced by acute or chronic pesticide exposure. It is important that DNA damage and oxidative stress have been proposed as mechanisms that could mechanistically link pesticide exposures.

In patients with clinically significant fibrosis, a2-macroglobulin concentrations also increase[28].

This acute phase protein inhibits matrix metalloproteinases, and its production by hepatocytes and activated stellate cells is up-regulated during fibrosis. Serum gamma globulin is associated with liver fibrosis and portosystemic shunts.

Chronic hepatitis is classified by histology based on activity of inflammation and degree of fibrosis; extent of fibrosis relates to likelihood of developing cirrhosis.

Albumin is commonly measured in patients suspected of progressing to cirrhosis. Although it is not as sensitive as other markers, it is used as a marker of severity as part of the Child-Pugh classification of cirrhosis.

Primary liver cancer (HCC) is a serious late complication of chronic hepatic injury, particularly in cirrhosis caused by HBV, HCV, and hemochromatosis.

The a-Fetoprotein (AFP) is more likely to be increased as the degree of hepatic fibrosis increases[29], especially in cirrhosis; AFP 17.8 mg/L has a sensitivity of 35%, a specificity of 98.6%, and a positive predictive value of 97.7% for cirrhosis[30].

Conclusions

Pesticides are ubiquitous contaminants of the environment and have been found in air, soil, water, and human and animal tissues in samples from all over the world. They include insecticides (arthropods), fungicides (fungi), herbicides (weeds), rodenticides (rats), molluscicides (snails), and others.

A pesticide is defined as any substance or mixture of substances intended for preventing, destroying, or controlling any pest, including vectors of human or animal diseases, unwanted species of plants or animals that cause harm during the production, processing, storage, transport, or marketing of food, agricultural commodities, wood and wood products, or animal feedstuffs, or which may be administered to animals for the control of insects, arachnids, or other pests in or on their bodies.

The improper use of pesticides may engender biological effects beyond those for which they were originally manufactured, also in view of the great interest aroused by these substances, suspected to be involved in the pathogenesis of neurodegenerative diseases (Parkinson's disease and progressive supranuclear palsy)[31-32].

The process of selection and validation of biomarkers for monitoring and screening requires careful consideration of the relevance and accuracy of the tests.

During risk assessment of exposure to pesticides, it is important to consider the following important issues[33]:

• Biomarkers are predictive assays rather than diagnostic ones.

• Humans are not exposed uniformly to single, pure chemicals, but rather to complex and variable mixtures of substances; some of these substances have antimutagenic activity while others may interact synergistically.

• Humans possess a number of metabolic

processes that may eliminate, detoxify, or possibly activate certain chemicals. Furthermore, human populations are heterogeneous with respect to mutagen sensitivity.

Chronic liver disease is a major risk of pesticides exposure. The laboratory tests have a variety of potential uses in the diagnosis of liver diseases. Routinely performed tests (e.g., serum bilirubin, albumin, and prothrombin time) may be of prognostic value in liver disease but they are not accurate in identifying early fibrosis. The most promising markers of fibrosis relate to collagen deposition, such as serum P-III-P and tissue inhibitor of metalloproteinase. Currently available laboratory tests are of value in excluding various types of liver diseases. Commonly performed serum liver enzymes are of limited value in monitoring liver diseases[34].

The development and application of laboratory tests that can identify early fibrosis and chronic hepatic disease have the potential of reducing healthcare costs and suffering associated to chronic liver diseases.

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Request reprints from:
Prof. LIBORIO RAMPELLO
Direttore U.O.C. di Neurofisiopatologia
Padiglione 2 (Neurologia)
Policlinico dell'Università di Catania
V. Santa Sofia 78
95100 Catania
(Italy)