The impact analysis of toxicological studies of 2, 4-D exposure on humans and other non-target organisms

Kavita Sharma[1], Rajesh[2], Sudhir Kumar Kataria[3]*
[1,2,3]Department of Zoology, Maharshi Dayanand University, Rohtak-124001, India

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ABSTRACT *The chlorophenoxy herbicides such as 2, 4-Dichlorophenoxyacetic acid (2, 4-D) although have important place in field of agriculture but their effects on health of farmers and other groups is not negligible. The general population is exposed to the residues of herbicides, including physical and biological degradation products in air, water and food. Occupational exposure occurs at all stages of herbicide formulation, manufacture and application of herbicide and during the storage procedure.2, 4-D is a plant growth regulator and its formulations include esters, acids, and several salts, which vary in their chemical properties, environmental behavior, and to a lesser extent, toxicity. Biological monitoring provides a useful tool to estimate the genetic risk deriving from an integrated exposure to different chemicals or a mixture of such chemicals. The various agrochemical ingredients were reported to induce mutations, chromosomal alterations or DNA damage. Although a number of biomarkers are available to assess transient and permanent genotoxic responses, biomonitoring studies on human populations exposed to pesticides have essentially focused on cytogenetic end-points, namely chromosomal aberrations (CA), micronuclei (MN) frequency and mitotic index (MI).This paper is generally focused on comprehensive study of the various reports on mechanism of action of 2, 4-D and in vivo and/or in vitro genotoxicity and cytotoxicity caused due to exposure with 2, 4-D inanimals and human population. Induction of oxidative stress due to 2, 4-D exposure is accompanied by unregulated generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl radicals and singlet oxygen which generally associated with cell damage and causing cancer. Results compiled here suggested that exposed group is more prone to chromosomal damage instead of non-exposed group to 2, 4-D accept some negative reports. Studies related to 2, 4-D exposure in human and animals showed the genotoxic and cytotoxic potential of this herbicide and suggested the need of more safe and controlled use of herbicides particularly 2, 4-D.*

Keywords: *2, 4-D, chromosomal aberrations, genotoxicity, oxidative stress, ROS, sister chromatid exchange.*

2, 4-D: A GENERAL VIEW

Most regulatory agencies mainly depend on toxicological reports, so the results of *in vivo* or *in vitro*studies are becoming more important in the field of human health. For this reason and generally for public health, analysis of the epidemiologic studies on crop protection products is of great value. Pesticides refer to the substances which are meant for attracting, seducing, and then destroying any pest. Pesticides are a class of biocide. These protect plants from damaging influences such as weeds, fungi, or insects. The pesticides are categorized in herbicides, insecticides, nematicide, termiticide, molluscicide, piscicide, bactericide, animal repellent, fungicide and sanitizer.

The herbicide, 2, 4-Dichlorophenoxyacetic acid (2, 4-D), is an example of a pesticide for which the epidemiology data are continually reviewed and debated. Recently re-registered for use by Health Canada (2008), and the US Environmental Protection Agency, EPA (2005), 2, 4-D is currently being re-evaluated by the European Union (Burns and Swaen, 2012). Garabrant and Philbert (2002) observed more strongly that the exposure to 2, 4-D is associated with cancers like STS (soft tissue sarcoma), NHL (non- Hodgkin lymphoma) or HD (Hodgkin's disease).The chlorophenoxy herbicide, 2, 4-D, has been registered for usesince the 1940s. As a selective herbicide, 2, 4-D is usedto control broadleaf weeds in crops, gardens, lawns, forests and aquatic settings.In aerobic conditions, 2, 4-Ddecays rapidly from2 to 13 days (Wilson *et al*., 1997). Whereas in case of humans, 2, 4-D isexpelled out from the body as unmetabolized form in urine with a half-life of 10 to33 hours, an average of 17.7 hours (CDC, 2009; Sauerhoff*et al*., 1977). 2, 4-D is excreted in urine of both animalsand humans by an organic anion transporter,OAT-1; toxicity of 2, 4-D in rodents is typically limitedto dose levels that saturate renal clearance (>50 mg/kg/day). Toxicity observed in rodents at doses above renalsaturation is generally not regarded as relevant to humanhealth risk (Timchalk, 2004; EPA, 2005). Chronic toxicityof 2, 4-D has been tested in laboratory animals at a widerange of dose levels (Garabrant&Philbert, 2002; EPA,2005). Studies in both rats and mice have shown no carcinogeniceffect of 2, 4-D and the US EPA classifies 2, 4-D as aGroup D chemical (not classifiable as to human carcinogenicity). But in 2015, IARC monographs classified 2, 4-D as "possibly carcinogenic to humans" (Group 2B)(Burns and Swaen, 2012). As oneof the most widely used herbicides in the world, 2, 4-D continues to be one of the most studied pesticides, bothin animals and in humans.Ideally herbicides kill certain targets while leaving the desired crop relatively unharmed. Absolute selectivity however is difficult to achieve and most herbicides

are supposed to be toxic to human as well. Bolognesi (2003) concluded that all people are necessarily exposed to herbicides, through environmental contamination and occupational use. The general population is exposed to the residues of herbicides, including physical and biological degradation products in air, water and food. Occupational exposure occurs at all stages of herbicide formulation, manufacture and application of herbicide and during the storage procedure. Moreover, inert ingredients have no pesticidal activity; they may be biologically active and sometimes the most toxic component of an herbicide formulation. Herbicides have been declared to cause various health effects ranging from skin rashes to death. Bolognesi (2003) concluded that herbicides are the most important method in self-poisoning in the developing world. Three million cases of herbicide poisoning in which nearly 220,000 fatal, occur all over the world every year. In emerging countries, where there is insufficient regulation, lack of surveillance systems, less enforcement, lack of training, inadequate or reduced access to information systems, poorly maintained or nonexistent personal protective equipments, and larger agriculturally based populations, the incidences are expected, then, to be higher. Soloneski*et al*. (2011) concluded that the number of poisonings increases dramatically in developing countries where the marketing of herbicides is often uncontrolled or unlawful. And also in these countries the misbranded or unlabeled formulations are sold at open stands. The herbicide industry in the developed world has made some progress in the field of development and production of low risk and environment friendly herbicides formulation. But in the developing countries still now herbicides are mainly available in conventional formulations such as dust, wettable powder, emulsifiable concentrates and solutions etc. Improper and unsafe use is very common in India. 2, 4-D is known from its use as a compound (together with 2, 4, 5-T) Agent Orange in the Vietnam War. At that time, the most visible detrimental effects on human health were caused by dioxin, which is a highly toxic byproduct.

2,4-D was detected in stomach, blood, brain and kidney of 4-day-oldrat neonates fed by 2, 4-D exposed mothers (Sturtz*et al*., 2000).Hoar *et al*., 1986 studied 170 males with NHL reported that farming and phenoxy herbicide use were associated with a risk of NHL when non-farmers were used as a comparison. Woods *et al*., 1987 performed a population-based study in Washington reported that among 576 male NHL cases and 694 male controls, there was an association between the disease and farming.

The production and degradation of 2, 4-D leads to thecreation of many compounds likechlorophenols (Bukowska and Kowalsk, 2003; Michalow, 2005) or dioxins (Bukowska*et al*., 1998; Bukowska, 2000) that exert strong toxicity. In theperiod from 1962 to 1999, 66 cases of phenoxyaceticherbicide poisoning were noted, including 22 casesthat ended in the patient's death (Bradberry*et al*., 2000). Persons employed in production, commercial distribution, packaging and repackaging as well as other plant protection personnel and those involved in plants spraying are chronically exposed to phenoxyherbicides action (Burns *et al*., 2001; Phillips and Boder, 2004). Children living in agricultural communities are heavily exposed to pesticides, whether or not they work in the fields (Luc *et al*., 2000; Nishioka*et al*., 2001). Farmers' children come in contact with pesticide residues from their parents' clothing, dust tracked into their homes, contaminated soil in areas where they play. They are contaminated by food from fields where 2, 4-D is used, also by aerial spraying, contaminated well water, and breast milk (Waited *et al*., 2005).According to Bukowska (2005), 2, 4-D can cause low growth rates, reproductive problems, changes in behavior, or death in non-tagged species. 2, 4-D is easily absorbed into the human organism from the alimentary tract and skin and is subsequently excreted in the urine in nearly unchanged form.

MODE OF ACTION AND CHEMISTRY OF 2, 4-D:

The basic form of 2, 4-D is 2,4-dichlorophenoxyaceticacid, but it is often formulated as an inorganic salt, amine or ester (WHO, 1984). The two primary approaches to constructing 2, 4-D are condensation of 2.4-dichlorophenol with monochloroacetic acid or chlorination of phenoxyacetic acid (WHO, 1984). 2, 4-D alkali metal salts are prepared by reaction of 2, 4-D with a metal base, 2, 4-D amine salts by reaction of 2,4-D with amine, and 2, 4-D esters by acid-catalyzed esterification or direct synthesis of a monochloroacetic acid ester with dichlorophenol (WHO, 1984). Plants absorb 2,4-D through their roots and leaves within 4-6 hours after application. Within the plant, 2, 4-D acts like the auxins or plant growth-regulating hormones and stimulates growth, rejuvenates old cells, and overstimulates young cells, which leads to an abnormal growth pattern and death in some plants (Mullison, 1987). Plant metabolism also is affected by 2, 4-D through modification of enzyme activity, respiration, nucleic acid synthesis, protein synthesis, and cell division (EPA, 1989), and through congestion of the phloem, thus interfering with food transport (Mullison, 1987). 2,4-D is selectively toxic to broad leaf plants due to their larger leaf area, which leads to absorption sufficiently to affect plant growth (Seiler, 1978). 2,4-D was first registered for use in the U.S.in 1948 (EPA, 1989). By 1983, approximately 1500 products containing 2,4-D, were registered with the U.S. Environmental Protection

Agency (Easley*et al*., 1983). According to Archibald and Winter (1990), the annual use of the 2,4-D active ingredient in the U.S.was estimated in 1990 to be 52 to 67 million pounds. 2,4-D is a keystone in agriculture, forestry, and lawn care for weed removal. It is estimated that over 75% of the total usage of 2, 4-D in the U.S. is for weed control in agriculture, especially in wheat and corn fields (EPA, 1989). Additional uses of 2, 4-D is in forestry, along rights-of-way, on rangelands, in parks, on golf courses, in aquatic situations, and, to a much lesser extent, for home lawn care and gardening (Ibrahim *et al*., 1991). 2,4-D is used very rarely as a growth regulator (Munro *et al*., 1992). Examples of this type of use include prevention of premature dropping of fruit, favourable selection of the growth of medium-sized potatoes, enhancement of the colour of potatoes (WHO, 1989). Increased size of citrus fruits, and increased vitality of citrus fruits after harvest to retard fungal growth (WHO, 1975).

2, 4-D AND OXIDATIVE STRESS INDUCTION:

Although 2, 4-D has been reported to be a proliferator of perosixomes (Bradberry*et al*., 2000) and inducer of mitotic and meiotic disruptions/abnormalities both *in vivo* and *in vitro*in plant cells (Khalatkar and Bhargava, 1982). Rivarola*etal.* (1985) showed that in mammalian cells *in vitro*, 2, 4-D inhibits cell growth, protein and DNA synthesis, and arrests cells in the G/S phase of the cell cycle. Some researchers suggested that herbicide like 2, 4-D induce oxidative stress in cell system (Banerjee *et al*., 1999; Martinez-Tabche*et al*., 2004 and Maire *et al*., 2007). Herbicide 2, 4-D has been suggested as a potential environmental endocrine disruptor and oxidative damage inducer in a number of studies (Munro *et al*., 1992; Mi*et al*., 2007).Selassie *et al*., (1998) suggested that generation of ROS is related to two properties, one is the formation of free radicals from them, andthe second is a direct attack of these phenoxyl radicals on biochemical processes in various sensitive metabolic pathways. Beddowes*et al*. (2003) also proposed a mechanism for carcinogenicity that was the induction of oxidative stress leading to secondary genotoxicity. Induction of oxidative stress due to pesticide exposure is accompanied by unregulated generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl radicals and singlet oxygen. ROS are produced during normal process in the cell. Antioxidant systems of the cell minimize damage caused by ROS under normal conditions. But when ROS generation increases to an extent that it overcomes the antioxidant systems of the cell, the result is oxidative stress. *In vitro* reports examined the effects of 2, 4-D on hepatocytes and red bloodcells (Palmeira*et al*., 1995; Bukowska, 2003). *In vivo* oxidative activity in cells due to effect of pesticides has been studied in many species including yeast, plantsand rats (Romero-Pueratss*et al*., 2004; Teixeira*et al*., 2004). Celik *et al*.(2006) studied the effect of two doses i.e. 0.5mg/ml and 1mg/ml of 2, 4-D on serum enzymes and antioxidant system of rat tissues like liver, kidney, brain and heart. The study showed that serum AST, CPK, and LDH activities were significantly increased with both doses of 2,4-D. The lipid peroxidation end-product MDA significantly increased in the all tissues without any change in the brain anderythrocyte of rats treated with both the dosages of2,4-D. The GSH depletion in the kidney and brain tissuesof rats treated with both dosages of 2,4-D was found to be significant. Also, the GSH level in the liver was significantly depleted with 0.5mg/ml of 2,4-D, whereas the GSH depletion in the same tissue did not significantly change with the treatment. SOD activity in the erythrocytes, liver, and heart was either significantly decreased with two doses of 2,4-D. Although the CAT activity significantly increased in the erythrocyte and brain of rats treated with both doses of 2, 4-D but remained unchanged in the liver, heart, and kidney. However in case of plasma membrane, Rosso *et al*. (1998) demonstrated that there is no significant penetration of lipid monolayers by 2, 4-D at lower concentrations (0.022 mg/L) but higher concentrations (2.2–220 mg/L) increase lipid bilayer width and cause deep structural changes in the hydrophobic region of model membrane systems. These high concentrations have also damaged human erythrocyte cell membranes in a dose-dependent manner and cause change in shape to a spiny (echinocyte) configuration with numerous surface bulbs and/or spinules (Suwalsky*et al*., 1996). So the herbicide induced dose-dependent effect on plasma membranes may also be related with the dose-dependent toxicity of central nervous system (CNS). Only small amounts of herbicide were found in the brains of non-target animals administered 100 mg/kg or less (Kim *et al*., 1988) consistent with low concentrations having minimal effects on the plasma membranes which comprise the blood-brain barrier. In case of high dose exposure (250– 500 mg/kg) of herbicide, reversible selective damage to the blood-brain barrier occurred in rats (Hervonen *et al*., 1982). The intensity of herbicide-induced cerebro-vascular damage in rats increased in the order 2,4,5-T, MCPA, 2, 4-D however 2,4,5-T practically neither cause damage to the brain capillaries nor inducing extravasations of plasma proteins in brain of rat (Elo *et al*., 1988). In addition chlorophenoxy herbicides have also been shown to disrupt organic anion transport system in the choroid plexus that facilitates the removal of potentially toxic anions from the brain to the blood. Experimental studies have examined competitive inhibition and ultimately saturation of anion

exchange system by chlorophenoxy herbicides (Kim and Pritchard, 1993) due to which the herbicide and acidic endogenous neurotransmitter metabolites accumulate in the brain. In support of this mechanism, Kim *et al*., 1987 and Elo and MacDonald, 1989 demonstrated that homovanillic acid and 5-hydroxy-3-indoleacetic acid, metabolites of the neurotransmitters dopamine and serotonin accumulate in the CNS of rats following administration of 2,4-D.Another consequence of the disruption of plasma membranes is direct cytotoxicity. Due to its central role in the metabolism of xenobiotics, the liver is potentially vulnerable to the toxic effects of these herbicides (Bradberry *et al*., 2000). Some experimental in vitro and in vivo animal reports show no genotoxic potential for 2, 4-D whereas neurotoxicity studies showed aberrations in locomotion and open field behavior at higher concentrations saturating renal clearance but no histopathological changes in neural tissues. The neurotoxic changes were reversible and were at doses that exceeded general toxicity range.

The formation of acetyl-CoA is an important part inmany biochemical pathways such as the citricacid cycle, in the synthesis of fatty acids and lipids, and in the synthesis of cholesterol and then steroid hormones. Acetyl-CoA also reacts with cholineto produce the neurotransmitter, acetylcholine. Sastry *et al*. (1997) suggested that chlorophenoxy acids are relatively same in structure to aceticacid and are able to form analogues of acetyl-CoA (e.g.,2, 4-D-CoA) *in vitro*.Such analogues have the potential to disrupt several numbers of cellular metabolic pathways involving acetyl-CoA. For example, analogues can enter the acetylcholine (ACh) synthesis pathway by forming choline esters (e.g., 2,4-D-ACh) which may mimic cholinergic messengersat muscarinic and nicotinic synapses which account in the development of myotonia, a common symptom of chlorophenoxy herbicide poisoning in experimental animals(Bradberry *et al*., 2000). Chlorophenoxy derivatives of CoA could alternatively enter other acetyl-CoA metabolic pathways and interfere with energy metabolism and with the utilization of two-carbon fragments inthe citric acid cycle. Chlorophenoxy herbicides have been shown to alter cholesterol levels of serum and increase β-oxidation of fatty acids (Hietanen *et al*., 1985) perhaps also through this mechanism.

Chlorophenoxy herbicides may alter energy metabolismin mitochondria of rat liver by uncoupling oxidative phosphorylation (Palmeira *et al*., 1994) possibly by disruption of the phospholipidbilayer of mitochondrial membranes. 2, 4-D increases potassium influx in nervous tissue and myotonia maybe triggered through continued depolarization of the sarcolemma (Popham and Davies, 1964). ATP concentration reduction may also affect the operation of the sarcoplasmic reticulum Ca^{2+}-ATPase pump, causing irregularities in calcium regulation in muscle cells resulting in sustained muscle contraction. Many animal and human studies indicate that chlorophenoxy herbicides may cause interruption in the neuromuscular junction. This may arise due to interference in production of acetylcholine which directly affects plasma membranes causing failure of ion channels through uncoupling of oxidative phosphorylation where ATP depletion causes disturbed Ca^{2+} regulation in muscle.

EXPOSURE TO 2, 4-D AND TOXICITY:

Despite widespread use of these agents, few reports of systemic toxicity following inhalational and/or dermal exposure have been published. Each involved inadvertent spillage of 2,4-D on the limbs(Goldstein *et al*., 1959 and Todd, 1962), in the context of little or no protective clothing or safety precautions and no prompt skin decontamination. In several other cases, adverse effects have been reported following predominantly inhalational exposure. Interpretation of these cases is complicated by the fact that some undoubtedly involved dermal as well as inhalational exposure, and possibly also ingestion. Moreover, and appropriately, the etiological role of chlorophenoxy herbicides in many of these cases has been challenged (Mattsson and Eisenbrandt, 1990).

Goldstein *et al*. in 1959 described a 39-year-old farmer's case that experienced excessive cutaneous exposure to a 40% aqueous solution of the diethylamine salt of 2, 4-D while spraying in fields over 4 days and some inhalation may occurred. After completion of spraying, paresthesiae in the fingers and toes and myalgia was noticed by him. Over the next 2 weeks, he developed weakness and in coordination of the hands with reduced biceps, triceps, and ankle reflexes. Position, vibration, and light touch sensation were reported as impaired in the distal extremities but electromyographic (EMG) studies were normal. Clinical findings were of a flaccid paresis with impaired light touch, pain and temperature sensation, and reduced or absent reflexes. There was EMG evidence of denervation in 2 cases, described as ''minimal'' in one of these. Fasciculation affecting all limbs but without weakness was the principal feature in another case. All patients recovered although mild weakness was present at 2–3 year follow-up in 3 cases (Goldstein *et al*., 1959). Gastrointestinal and peripheral neuromuscular symptoms were also reported following occupational exposure in which inhalation was an important route of exposure either alone or in combination with

cutaneous exposure. Features described included nausea and vomiting, constipation, abdominal pain, limb paresthesiae and pain, myalgia, weakness, and hypertonia (Todd, 1962). In some cases, features persisted for several weeks after a single exposure. Dizziness, vertigo, and brief loss of consciousness also occurred. Cardiac features including intermittent nodal tachycardia, chest pain, and palpitation have also been reported rarely. In another case, Alix*et al*., 1974 described the development of pericarditis in a 43-year-old man who some 12 hours earlier had applied MCPA to a field. Clinical features resolved over some 2 weeks. The authors suggested an ''allergic'' etiology. Also, England (1981) reported that prolonged exposure to herbicides such as 2, 4-D has been associated with coronary artery ectasia (CAE). More than 50% of CAE cases were reportedly caused by atherosclerosis (Lin *et al*., 2008). The precise mechanism of 2, 4-D acute toxicity is not elucidated but may involve disruption of plasma and intracellular membranes or uncoupling of oxidative phosphorylation (Bradberry *et al*., 2000); this last mechanism may be involved in the generation of oxidative stress. In fact, many studies have implicated oxidative damage as the central mechanism of pesticide toxicity (Gutteridge & Halliwell, 2000).

Kang *et al*. (2006) have noted that there are long-term health consequences of Agent Orange herbicide (a mixture of 2, 4-D and 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T)) exposure among army Vietnam veterans who were exposed to this herbicide. The study group showed significantly higher risk of diabetes, heart diseases and circulatory diseases including hypertension. Recently, Schreinemachers (2010) indicate that human exposure to 2, 4-D was associated with changes in biomarkers that have been linked to risk factors related to the pathogenesis of acute myocardial infarction and type-2 diabetes, such as dyslipidemia and impaired glucose metabolism. Anti-cholinesterase compounds are the most common method of poisoning in India but herbicide poisoning is also a method of suicide and is associated with high death rate. Researchers compiled the medical cases of 69 people from1962 to 2004 who had ingested 2, 4-D and other chlorophenoxy herbicides and reported death in 23 patients. Recently in a poisoning case report of a 33-year-old female ingesting 70 ml liquid containing 58% of 2, 4-D-dimethylamine salt, the described symptoms of intoxication include nausea, vomiting, abdominal pain, hepatic injury and kidney injury, hypertonia, areflexia, depression of central nervous system, fasciculation and coma, hypotension, and ECG changes(Hiran and Kumar, 2017).The poison with 2, 4-D is rare and it does not have any antidote. Its toxic effects involve heart, central and peripheral nervous system, liver, kidneys, muscles, lungs and endocrine system (Bradberry*et al*., 2000). The early recognition and urine alkalization with high flow urine may save the patient. 2, 4-D is a poison which carries a high mortality (Hiran and Kumar, 2017).

TOXICITY IN ANIMAL SYSTEM:

However the necessity of the use of herbicide can't be neglected but its effects on non-target organisms like rodents, birds, fishes etc. are also of a great concern. Free radicals generated due to the metabolism of 2, 4-D interfere in the cell system of the animals and produce mutagenic and clastogenic effects in their genome which may lead to depletion of the species or generation of harmful genome for example in a study conducted by Venko*et al*., 2000 showed the effect of 2, 4-D herbicide by using three test systems i.e. yeast, transformed hematopoietic and mouse bone marrow cells. The results obtained in this study demonstrated that 2, 4-D has cytotoxic and mutagenic effects.The positive response of yeast and transformed hematopoietic cells was verified in kinetics and dose-response experiments. The analysis of metaphase chromosomes indicated a statistically proved induction of breaks, deletions, and exchanges after the intraperitoneal administration of 2, 4-D in mice.

In 2001, Amer and Aly studied the effect of 2, 4-D and its metabolite 2, 4-DCP in bone marrow, germ cells and sperm head abnormalities in mice treated orally with different concentrations (1.7, 3.3 and 33 mg/kg BW) of these compounds and showed a significant increase in percentage of chromosomal aberrations in bone marrow and sperm cells at all concentrations in comparison with control group used during the study.

In an another study conducted by Bujaidar*et al*. (2001) showed the effect of 2, 4-D in somatic and germ cells of mice by an oral administration to three groups of mice (50,100 and 200 mg/kg BW) in comparison with control group of animals administered with distilled water, pH 10.5 and another group injected with cyclophosphamide (50 mg/kg BW). In somatic and germ cells, the results showed a significant increase in SCE with the two high doses tested in a response that was manifested in a dose-dependent manner whereas cyclophosphamide induced cytotoxic damage and a cell-cycle delay. Their results showed that 2, 4-D is a moderate genotoxicant in mice treated in vivo with high doses, and suggests a minor hazard for humans in that conditions of its use.

According to Arias, 2003 the commercial formulation of 2, 4-D and pure 2, 4-D both at the concentration of 4mg/embryo induced a dose-related increase in SCE frequency in 4-day old chick embryos. Significant cell cycle progression inhibition was noted in dose related manner.

Celik *et al.,* 2006 studied the effect of 2, 4-D on biochemical parameters of rats and demonstrated that serum AST, CPK, and LDH activities were significantly increased with 2, 4-D. The lipid peroxidation end-product MDA (Malondialdehyde) significantly increased in the all tissues treated with both dosages of 2,4-D. Also, the GSH level in the liver was significantly depleted with 50 ppm of 2,4-D. Whereas the SOD activity in the erythrocytes, liver, and heart was either significantly decreased or not changed with doses of 2, 4-D. Wafa*et al*., 2013 hypothesised and suggested that at higher doses in rats, the 2,4-D exert its cellular action by the induction of oxidative stress and lipid peroxidation. Sub-acute levels of 2, 4-D resulted in a state of liver injury and extensive oxidative damage in rats as manifested by the significant changes ($p<0.05$) in these enzymes. So on the evaluation of antioxidant enzyme activities compared to the control, SOD activity was demonstrated to have increased significantly at15 mg/kg of 2, 4-D, and to have decreased significantly for 75and 150 mg/kg of 2, 4-D. Furthermore, a significant decrease was observed in the CAT, GPx and GR activities for the threestudied doses of 2, 4-D ($p<0.05$).

TOXICITY IN HUMAN BLOOD LYMPHOCYTES AND OTHER MAMMALIAN CELL CULTURE SYSTEMS:

The use of herbicides in agricultural fields can cause health problems due to their persistence in the environment and accumulation throughout the food chain or dermal exposure and inhalation during the spraying of herbicides. Exposure to these chemicals results in induction of aberrations in cells which ultimately induce mutations in human genome. Several studies have been conducted to evaluate the clastogenic and mutagenic potential of 2, 4-D in cultured lymphocytes.Korte and Jalal (1982) evaluated the potential of 2, 4-D to be mutagenic in cultured lymphocytes. Chromosomal damage, though statistically insignificant, occurred at doses as low as 0.2µg/mL and increased at a statistically significant level at concentrations of 50µg/ml or higher. Potential mutagenicity, based on rates of increase in SCE, was significant at 10µg/ml or higher concentrations. Whereas Linnainmaa (1983) in an *in vivo* study in forestry workers spraying foliage with either 2, 4-D, MCPA, or a mixture found no induction of SCEs in peripheral lymphocytes. In another study, Turkula and Jalal (1985) observed increase in SCE in peripheral human lymphocytes exposed *in vitro* at 50, 100, and 250µg/mL, which were statistically significant at the lower doses, and the increase was noted less at higher doses than the lowest dose. Mustonen*et al*. (1986) evaluated chromosomal aberrations *in vivo* in lymphocyte cultures from 19 exposed 2, 4-D and MCPA Swedish forestry sprayers. Workers sprayed 333 g/L 2, 4-D and/or 167 g/L MCPA during spraying period for a minimum of six days and a maximum of 28 days. No increase in chromosomal aberrations in the lymphocytes of workers was observed in this study. These authors also conducted an *in vitro* study in which human peripheral lymphocytes were cultured with different concentrations of pure 2, 4-D as well as a commercial herbicide containing 2, 4- D. The pure 2, 4-D product showed no induction of chromosomal aberrations of any kind, but the commercial mixture showed statistically significant differences from controls in a dose-dependent manner starting at 0.5mM (110ppm). The authors suggest that this was due to impurities and phenols contained in the commercial mixture. Whereas, Clausen *et al*. (1990) and Jacobi and Witte (1991) in separate studies involving a commercial formulation of 2, 4-D argued that observed differences in toxicity may be due to differences in chemical structure between the pure compound and the soluble salt. Kiaoumova and Khabutdinova (1998) investigated the effect of dioxin-containing products on the cytogenetic characteristics of peripheral blood lymphocytes of herbicide plant workers in Ufa. They found that the mean incidence of cells with chromosomal aberrations was twofold higher in the herbicide plant workers than the mean incidence level of controls groups consisting of people with no professional contact to herbicides or hospital stuff working in the close vicinity of the herbicide plant in Ufa (for both cases: $p<0.05$). Moreover, the mean chromosomal aberration cell incidence in the controls groups was also two times higher than the average level of spontaneous aberrations in humans. The chemical herbicides 2,4,5-trichlorphenol(2, 4, 5-T) and 2,4- dichlorophenoxyacetic acid (2, 4-D) appeared to affect various cellular cycle phases. Chromosomal type aberrations occurred in the Go stage of cellular cycle and chromatic type aberrations in the G2 stage. In the S stage, the aberrations of both types were observed. Our results indicate that the herbicides 2, 4,5-T and 2, 4-D have mutagenic effects in humans.

Gollapudi*et al*., 1999 investigated the genetic toxicity of an ester (2, 4-D 2-butoxyethylester) and two salts (2, 4-D isopropylamine and 2, 4-D triisopropanolamine) in cultured mammalian cells. The end points used were the induction of chromosomal aberrations in primary cultures of rat lymphocytes and forward mutations at the HGPRT locus of Chinese hamster ovary cells. There was no evidence of genotoxicity for the test materials in the experimental systems used. Zeljezic and Garaj-Vrhovac (2001) considered the workers employed in three different pesticide production units at the same. The average duration of their

employment in pesticide production was 22-25 years (range 4–30 years). During production all subjects were simultaneously exposed to a complex mixture of pesticides (atrazine, alachlor, cyanazine, 2, 4-dichlorophenoxyacetic acid and malathion) spending the same amount of time in each of the three production units. Regardless of the period of sampling, in the exposed group statistically significantly increased numbers of aberrant cells, chromatid and chromosome breaks, acentric fragments and dicentric chromosomes compared with the controls were found. After the workers had spent 8 months out of the pesticide exposure zone the number of aberrant cells and all types of chromatid and chromosome aberrations decreased significantly compared with sampling after the high exposure period, but it still remained significantly higher in comparison with the control group.

Garry *et al*., 2001 studied 24 applicators and 15 minimally exposed foresters (control subjects). Chromosomal translocations, inversions, deletions (TIDs), breaks, and gaps occur more frequently among applicators who apply more than 1,000 gallons of herbicide during the application season. Most of these men are aerial applicators who apply a broad spectrum of herbicides including 2, 4-D. With regard to the possible relationship between urinary concentrations of 2, 4-D and chromosome aberrations, regression analyses indicated non-significant, negative regression coefficients. Garaj-Vrhovac and Zeljezic (2002) in a synergic study of pesticide sprayer exposed to a mixture of pesticides (atrazine, alachlor, cyanazine, 2, 4-Dichlorophenoxyacetic acid, malathion) reported a significantly increased number of chromatid and chromosome breaks, as well as the presence of dicentric chromosomes and chromatid exchanges in exposed subjects compared with control subjects (P <0.05), in human lymphocytes. In the exposed group, besides the increase in the number of chromatid and chromosome breaks and acentric fragments, dicentric chromosomes and chromatid exchanges were manifested. Results suggested that long-term occupational exposure to pesticides could cause genome damage in somatic cells and therefore may represent a potential hazard to human health.

Zeljezic and Garaj-Vrhovac (2004) also reported that 2, 4-D and their pesticide preparation Deherban caused an increase in chromatid and chromosome breaks, number of micronuclei and number of nuclear buds. The incoherent genotoxicity results may be attributable to different methodologies and treatment protocols. For example, the selection of compositionally different 2, 4-D salts and acids or solvents in each experiment could lead to different absorption and metabolism rates. The same way, doses, type of cells and organisms and also employed methods seem to be important. Generally, the recent year's reports (beyond 2000 with using the newest and more sensitive methods) confirmed genotoxic proprieties of 2, 4-D and those could be considered as the ultimate. Gonzalez *et al*. (2005) evaluated the genotoxicity of the 2, 4-D and a commercially-used derivative, 2, 4-D dimethyl amine salt (2, 4-D DMA) in CHO cells using SCE and single cell gel electrophoresis (SCGE) assays. Log-phase cells were treated with 2.0–10.0μg/ml of herbicides and harvested 24 and 36 h later for SCE analysis. Both agents induced significant dose-dependent increases in SCE, regardless of the harvesting time (2, 4-D: r = 0.98 and r = 0.88, P < 0.01, for 24 and 36 h harvesting times; 2,4- D DMA: r = 0.97 and r = 0.88, P < 0.01, for 24 and 36 h harvesting times). Neither test compound altered cell-cycle progression nor proliferative replication index (P > 0.05), but the higher doses of both compounds reduced the mitotic index of cultures harvested at 24 and 36 h (P < 0.05). Chiu *et al*. (2006) found a consistent relationship with respect to dieldrin, lindane, and toxaphene exposures, but no relationship with chlorophenoxy compounds, suggesting that although the evidence is increasing that this particular chromosomal aberration is significant with respect to NHL etiology, there is little support for a causal role for chlorophenoxy compounds in general and specifically 2, 4-D.

Soloneski *et al*. (2007) explored the genotoxic potential of 2, 4-D and its commercial derivative 2, 4-D DMA by measuring sister chromatid exchange (SCE), cell cycle progression, and mitotic index in human whole blood (WBC) and plasma leukocyte cultures (PLC). Cells were exposed to concentrations of 10, 25, 50, and 100μg/mL for 72 h. SCE frequency was statistically significant increased at concentrations from10 to 50μg/mL for 2, 4-D and from 25 to 100μg/mL for 2, 4-D DMA and in PLC, there was no increase in SCE. A significant delay in cell proliferation was observed in WBC after treatments with 25 and 50 μg/mL 2, 4-D and 50 and 100μg/mL 2, 4-D DMA, whereas in PLC, only 100μg/mL 2, 4-D altered cell-cycle progression. For both compounds, a dose-related inhibition of mitotic activity was observed. The results showed that the presence of erythrocytes in the culture system increase DNA and cellular damage induced due to 2, 4-D and 2, 4-D DMA. However, again these concentrations are high relative to environmental exposures. The US EPA has reviewed the potential genotoxicity and mutagenicity of 2, 4-D (USEPA 1994 and 1997), most recently in 2012. Those data show no evidence for heritable mutagenic effects in mammals but some evidence supporting 2, 4- D's potential to cause genotoxic effects.

Wafa *et al*. (2012) stated that several researchers use biological lipid membranes model like erythrocyte ghosts as they are sensitive to the peroxidative process; since they are rich in polyunsaturated fatty acids in

their membranes, a class of compounds highly susceptible to lipid peroxidation. Toxic influence of 2, 4-D may provoke disturbances in bilayer phospholipid structure that plays an important role in the correct function of cell membrane. Phenoxy herbicides interact with proteins and lipids of erythrocyte membrane (Suwalsky and Berites, 1996). Indeed, Janik and Wolf (1992) have demonstrated the inhibitory effect of chlorinated compoundson the Ca-ATPase which indicates a toxic effect to human erythrocytes functions. Bukowska*et al*. (1998) have found the increase in the level of methemoglobin (metHgb) and the change of the oxygen affinity of haemoglobin under the influence of 2, 4-D. Bukowska (2003) reported that treatment of human erythrocytes *in vitro* with 2, 4-D at concentration of 250and 500ppm resulted in decreased levels of reduced glutathione, decreased activity of superoxide dismutase, and increased levels of glutathione peroxidase. These significant changes in antioxidant enzyme activities and evidence of oxidative stress indicate that 2, 4-D should be taken seriously as a cytotoxic and potentially genotoxic agent. In 2008, Bukowska *et al*. present the evidence for a direct pro-oxidant activity of phenoxy herbicides. In fact, the pro-oxidative action of these compounds is strongly dependent on the localization of the substituent in the phenol ring. Also, they much more easily penetrate the cell membrane. The author proposed a metabolic reaction chain that explains the mechanism of action of 2,4-D *in vivo*. The authors have noted that the pro-oxidative capability of this herbicide is related with its hydrolysis to 2, 4-Dichlorophenol that may generate radicals oxidizing H2-DCF, marker of oxidative status of the cells. Other *in vitro* studies, dealing with the induction of oxidative stress after 2, 4-D exposure, were conducted on hepatocytes. Palmeira *et al*. (1994) suggested that 2, 4-D can decrease ATP, GSH and NADH levels while conversely increasing the levels of AMP, NAD, LDH and GSSG in rat hepatocytes. This herbicide at (1- 10 mM) may induce cell death by decreasing cellular GSH/GSSG ratio, promoting loss of protein thiol contents and inducing lipid peroxidation (Palmeira*et al*., 1995). In fact, it is suggested that membrane protein thiols can be attacked by radicals, resulting in a membrane protein thiol loss which in turn may also be associated with the development of hepatocellular injury.

Table-1: Summary of the *in vivo* and/or *in vitro* studies on chromosomal distortions of 2, 4-D on mammalian cell system.

Sr. no	References	Pesticide used/type of study	Subjects/ Health conditions/ Age	Toxicological parameter used	Results	Conclusions
1	Korte and Jalal, 1982	2, 4-D/*in vitro*	04/ healthy males	CA	Chromosome aberration occurs at higher frequencies and statistical significant at 50 µg/ml or higher.	Study reflects significant clastogenic effect of 2, 4-D
2	Mustonen et al., 1986	Pure and commercial (55%) 2, 4-D/*in vitro* and *in vivo*	6 (non-smokers) + 9 (smokers)=15 control and 10 (smokers) + 9 (non-smokers)=19 exposed subjects/healthy males/	CA	Commercial 2, 4-D induced aberrations significantly with concentrations of 0.500, 1.000 and 1.250 mM, in particular the number of chromatid type breaks was elevated where as pure acid induced aberrations non-significantly.	Genotoxic effects were noticed in case of *in vitro* study.
3	Kaioumova and Khabutdinova, 1998	2,4-D, 2,4,5-T/*in vivo*	19/exposed male and females/28-60 years	CA	The mean CA cell incidence of 4.47±0.75 among the workers of exposed group significantly exceeds the incidence level of the two control groups ($p<0.05$).	2, 4-D exhibited a mutagenic effect on humans.
4	Zeljezic and Garaj-	atrazine, alachlor, cyanazine, 2, 4-	40 (20 control and	CA	Positive and significant results	Concluded that 2, 4-D induce

	Vrhovac, 2001	dichlorophenoxyacetic acid and malathion/ *in vivo*	20 exposed subjects)/males and females/25-50 years		were noted when compared with control group. No effect of smoking, age and gender of subject were found in between- group variations in number and type of structural changes of chromosome.	the genotoxicity and clastogenicity.
5	Garry et al., 2001	2, 4-D/ *in vivo*	39/healthy males/30-50 years	CA	Induced chromosomal aberrations at higher concentration.	Showed clastogenic properties.
6	Garaj-Vrhovac and Zeljezic, 2002	atrazine, alachlor, cyanazine, 2, 4-Dichlorophenoxyacetic acid, malathion/ *in vivo*	30/healthy males and females/25-42 years	CA	Average %age of chromosomal aberrations in exposed group was higher (7.80%) than control group (1.02%) with significant increase ($p<0.005$).	Concluded that long term occupational exposure to pesticide cause genome damage
7	Zeljezic and Garaj-Vrhovac, 2004	2, 4-D/ *in vitro*	2/healthy non-smoker males	CA	Significantly increase chromosomal aberrations in exposed samples with concentration of 0.4 and 4µg/ml of 2, 4-D.	Could be genotoxic to human lymphocytes.

Table-2: Summary of the *in vitro* studies on genotoxicity of 2, 4-D on mammalian cell system.

Sr.no.	References	Pesticide used/type of study	Subjects/ Health conditions/ Age	Toxicological parameter used	Results	Conclusions
1.	Korte and Jalal, 1982	2, 4-D/*in vitro*	04/ healthy males	SCE	The mean frequency of SCE in normal human lymphocyte culture is 6.37. A significant increase in the frequency of SCE in 2, 4-D treated cells compared to the control is attributed to the mutagenic effect of the compound. The proportion of SCE increased with the increased concentrations of 2,4-D.	Study reflects significant mutagenic effect of 2, 4-D
2.	Linnainmaa, 1983	2, 4-D and MCPA/*in vivo*	50/exposed healthy males/20-60 years	SCE	No induction of SCE significantly due to direct action of 2, 4-D and MCPA as compared to control subjects.	Suggested that 2, 4-D had genotoxicity due to indirect mode of action and could be analyzed on the basis of structural chromosomal changes.
3.	Turkula and Jalal, 1985	2, 4-D/*in vitro*	Not mentioned	SCE	A 50 µg/ml dosage caused a highly significant increase in SCE. Dosages of 100 and 250 µg/ml elevated the rate of SCE, but not significantly.	In some extend 2, 4-D shows genotoxic effect on regular and long use at higher concentrations.
4.	Gonzalez et al., 2005	2, 4-D/ *in vitro*	CHO cells	SCE	Induced positive significant results regardless of harvest time of cultures	DNA damage provide evidence for genotoxicity
5.	Soloneski et al., 2007	2, 4-D, 2,4-D DMA/*in vitro*	06/ healthy males, non-smokers, non-alcoholic/≤30 years	SCE	Minimal genotoxicity was observed in PLC either after treatment with 2,4-D or 2,4-D DMA. For both test compounds, a slight increase in the SCEs frequencies was found although not reaching statistical significance when compared to control values ($p > 0.05$)	2, 4-D exerted a toxic effect on lymphocytes culture.

CONCLUSION:

The genotoxic damage induced by 2, 4-D appears to depend on the degree of exposure. Adose–response relationship can be hypothesised. Negative results have been associated with low levels of exposure with herbicide. Since workers are frequently exposed to complex mixtures of pesticides, it is difficult to attribute the genotoxic damage to any particular chemical class or compound. But relevant assessment of genotoxicity related to 2, 4-D exposure must be needed for betterment of the population living in 2, 4-D exposure prone area. Awareness about the proper use and effects of this herbicide related to the human health should be followed.

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