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CCL_4 INDUCED GENOTOXICITY AND PROTECTIVE EFFECT OF ANTIOXIDANTS AFTER *IN VIVO* ADMINISTRATION TO SHEEP

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Abstract

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Protective effect against carbon tetrachloride-induced genotoxicity was tested in sheep after antioxidative supplementation. Ten 3-4-year-old sheep were treated by carbon tetrachloride, orally. The total dose of 0.05 ml/kg b/w. was corresponding to the former recommended antihelminthic usage in the seventies. Five such sheep were pre-treated by injection of vitamin E (400 mg) and selenium (12 mg) subcutaneously 24 h prior to CCl_4 administration. Sister chromatid exchanges (as a DNA repair error detection technique) were used for genotoxic influences estimation. The technique was applied 16 days after CCl_4 administration in cultured peripheral lymphocytes. SCEs were significantly increased in both tested groups, i.e. with and without of antioxidative pre-treatment. However, the first group exhibited the significance of $p < 0.05$, contrary to the second untreated group in which the statistical significance reached $p < 0.001$ when using Student's *t*-test. This difference supports the protective genotoxic influence of the studied antioxidants in peripheral lymphocytes. In neither of the groups, a significant decrease in the proliferation index was recorded. Thus no cytotoxic effect on the investigated tissue has been proved. As our study showed, administration of a single concurrent treatment of vitamin E and selenium could reduce the genotoxic effect exhibited by the SCE frequencies in comparison to the non-protected individuals, remarkable.

Carbon tetrachloride, genotoxicity, SCE, vitamin E, selenium, protective effect

In ruminants, carbon tetrachloride CCl_4 has been widely used as an antihelmintic drug for a long time. Intoxication occurred in animals as a result of misuse of this substance by application of an overdose. Carbon tetrachloride as well as the other aliphatic hydrochlorides is actually used as a very effective organic solvent in various industrial branches. Its distribution and properties may lead to subsequent environmental contamination including the soil, the surface and surrounding waters (Rabergh and Lipsky 1996). Efficacy of the compound including various toxic influences, namely hepatotoxicity for the patients treated, has been demonstrated in different mammalian species, i.e. humans, guinea pigs and rabbits, such as skin irritation reactions (Wahlberg 1984), dose-related maternal toxicity and litter resorption in rodents (Narotsky and Kavlock 1995), immunosuppression due to a decrease of T lymphocyte populations in rodents or in sheep (Vajdovich et al. 1995).

With respect to cytotoxic, genotoxic or mutagenic activity of chlorinated aliphatic hydrocarbons the data showed that the DNA reactivity such of compounds increased with increasing degree of halogenisation (Tafazoli and Kirsch-Volders 1996). The carcinogenic potential of carbon tetrachloride has been early shown by Weisburger (1977) in mice in which liver and adrenal tumors were induced, and in rats in which disseminated neoplastic nodules were observed after oral exposure.

The positive clastogenic effect has been demonstrated by *in vitro* conditions in metabolic competent.MCL-5 and h2E1 human cell lines (Doherty et al. 1996) and in workers

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occupationally exposed to mixture of chlorinated solvents or carbon tetrachloride in comparison to the group that was not exposed (da Silva et al. 1997).

Because much of cellular or sub-cellular damage may originate due to lipid peroxidation-induced free radicals the particular effective mechanisms based in order to minimalize these nucleinic acid damaging reactions (Bell and Mehendale 1987). These changes could be moderated by the cytochrome P 450 inhibitors and antioxidants. Vitamin E as well as selenium influence is one of the most important free radical decreasing factors and corresponding health protective effects. Pre-treatment of experimental animals with vitamin E was shown to be effective against CCl_4 induced acute liver necrosis (Biasi et al. 1991; Parola et al. 1992).

In this paper we report the influence of antioxidants (vitamin E and selenium) on carbon tetrachloride induced sister chromatid exchanges (SCE) in sheep peripheral lymphocytes *in vivo.*

Materials and Methods

Healthy, 3-4-year-old non-pregnant Merino ewes were treated with non-lethal dose of 0.05ml/kg b.w. of carbon tetrachloride (99.8 % Microchem Bratislava, Slovak Republic) in 1:1 v/v dilution with paraffin oil via stomach tube into the rumen. However, one group of five sheep 24 was injected s.c. with E 400 mg s.c. as tocopherylacetatate and selenium 12 mg as a sodium selenite 24 hours prior to CCl_4 administration (vitamin E + selenium, Bremer Pharma GmbH , Bremerhaven, Germany). The serum concentrations of vitamin E and selenium ranged levels 2.82 ± 0.56 (spectrophotometric assay) and 0.173 ± 0.022 (determined by AAS model 410, respectively) prior to their experimental supplementation; thus the animals were not suffering from their latent insufficiency.

The experiments were aimed to detect clinically the protective effect of the antioxidants mentioned against the biochemical changes and health disturbances induced by CCl_4. Both experimental groups were housed at school farm at the standard conditions. Before and during the experiment, the same diet consisted of meadow hay *ad libitum* and BAK (concentrate for sheep) and fresh water was available *ad libitum*.

Cultivation of cells

Blood samples were obtained by jugular venipuncture into heparinized test tubes before, and on the 16th day following the administration of vitamin E and selenium to the second experimental group.

Lymphocyte cultures were prepared by adding 0.5 ml of heparinized whole blood from donor to 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 μM HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 μU/ml and streptomycin 250 g/ml), and phytohaemagglutinin (PHA, 180 μg/ml, Welcome, Dartford, England) at 38 °C for 72 h.

Bromodeoxyuridine (BrdUrd, Sigma) was added to all cultures at a final dose of 8 μg/ml 48h before the harvest. To achieve cytostatic block, colchicine (Merck, Darmstadt, Germany) was added at a concentration of 5 μg/ml 2h before the harvest.

Evaluation and statistical methods

Chromosome preparations were obtained by the standard method. Slides were stained with FPG technique to differentiate sister chromatids and cell cycles (Perry and Wolff 1974) for the SCE delumination and cell cycle kinetics. Fifty differentially stained metaphases per donor or total 250 metaphases per concentration were examined for the SCE. The proliferation indices (PI) were calculated according to Lamberti et al. (1983), thus frequencies of mitotic divisions (M_1, M_2 and M_{3+}).

Statistical analysis of results was performed using a simple analysis of variance (ANOVA). Then Student's *t*-test was applied to evaluate the SCEs significance for the both of the tested groups before and after the CCl_4 exposure. The same statistical procedure has been used with respect to the distribution of SCE among donors to evaluate the significant differences between treated with vitamin E and selenium, and untreated group.

Results

Results both of experiments focused on the CCl_4 in *vivo* genotoxicity in sheep are summarised in Table 1. Prior to statistic verification the ANOVA test was applied to detect the clastogenic effect of the compound tested. In all sheep treated with CCl_4 those missing the protective agent administration, the remarkable increasing of induced SCE was detected; $p < 0.01$ using the Student's *t*-test, for one individual minimally. The more significant performance of $p < 0.001$ has been obtained for a whole group of animals to compare to control level prior to CCl_4 administration.

Table 1
Sister chromatid exchanges and proliferation indices induced by CCl_4 administration with and without protective pre-treatment with vitamin E and selenium

Group	No of counted cells	SCE ± SD	PI
Control 1	250	6,94 ± 1.66	1.54
CCl_4	250	9,06 ± 2.34 ***	1.44 [a]
Control 2	250	6.88 ± 1.96	1.49
CCl_4 + vit. E and Se	250	7.33 ± 2.28 *	1.42 [a]

*, **, *** Significantly different values ($p < 0.05$, $p < 0.01$, $p < 0.001$: ANOVA, Student's *t* test
a - No statistical significance according to previous or χ^2 test
PI - Proliferation index

Table 2
Individual responses of donors to the CCl_4 administration with and without of protective pre-treatment with vitamin E and selenium

Group 1	SCE ± SD		Group 2	SCE ± SD	
Sheep No.	Control 1	CCl_4	Sheep No.	Control 2	CCl_4; vit E +Se
K 598	6.90 ± 1.23	8.98 ± 2.28**	K 30	6.90 ± 1.75	7.36 ± 2.27[a]
K 2142	6.76 ± 2.01	8.96 ± 2.41**	K 257	7.00 ± 1.98	7.40 ± 1.93[a]
K 334	7.10 ± 1.23	9.00 ± 2.28***	K 356	6.96 ± 2.10	7.32 ± 2.59[a]
K 542	7.00 ± 1.80	9.18 ± 2.36***	K 301	6.88 ± 1.75	7.48 ± 2.27[a]
K 2198	6.96 ± 1.78	9.20 ± 2.35***	K 326	6.68 ± 2.10	7.68 ± 2.59[a]

*, **, *** significantly different values ($p < 0.05$, $p < 0.01$, $p < 0.001$ Student's *t*-test)
a no statistical significance according to previous test

Contrary to this, none of the animals belonging to the second experimental group (i.e., pre-treated with vitamin E and selenium), exhibited a significant increase of SCE. The same criterion calculated for the experimental group, however was significant ($p < 0.05$). Kinetics both of experiments with respect to the individual response to CCl_4 administration is seen in Table 2.

According to Student's *t*-test, the protective effect for vitamin E and selenium against CCl_4 induced clastogenicity has been verified at the significance level of $p < 0.01$.

A slight, non-significant decrease in proliferation indices was also observed (Table 1). In both experiments, the proportions of M_1, M_2 and M_3 were not significantly different from those seen in controls. The difference in induced mitotic delay between the basic and post-experimental levels reflected by PI was not thus verified.

Discussion

In mammals, CCl_4 has been widely used as a model chemical in the study of acute lever injury and it is known to cause fat accumulation and necrosis in centrilobullar hepatocytes (Clawson 1989). *In vivo* studies with CCl_4 have indicated a species specific difference in the toxicity of this compound with mice being more sensitive than rats, and birds being least sensitive (Diaz Gomez et al. 1975).

With respect to its molecular characteristics CCl_4 does induce an increase of unsaturated fatty acid, lipoperoxide and free peroxide radical concentrations with the corresponding health disturbances.

In our experiment, one hour after CCl_4 exposure several sheep became anorectic with muscular fasciculation, convulsions and with increased respiratory rate. Within three hours, however, all sheep recovered clinically (Milad et al. 2000).

Genotoxic activities of CCl_4 have been reported in various assays. As for clastogenic effect of CCl_4 the micronucleus assay *in vivo* was widely employed. No increases in micronuclei was obtained in mouse bone marrow and mouse peripheral lymphocytes in comparison to the control after administration a single intraperitoneal injection of carbon tetrachloride (Suzuki et al. 1997; Crebeli et al. 1999). However, this substance was confirmed to cause cancer in rodents after prolonged exposure to CCl_4 (Westbrook-Collins et al. 1990). Positive clastogenic or aneugenic results after treatment with of CCl_4 were found in human metabolically competent cell lines; MCL-5 CYP which expresses cDNA encoding the human CYP1A1, and h2E1 cell lines containing cDNA for CYP2E1 (Doherty et al. 1996). Carbon tetrachloride in combination with chlordecone was genotoxic using the *in vivo – in vitro* animal model and a battery of biochemical assays to measure the DNA repair in the rat hepatocytes (Ikegwuonu and Mehendale 1991).

Because of long-lived tissue contacting different organs and organic systems, and that they have a potential to accumulate DNA lesions (Carrano and Natarajan 1988) the peripheral lymphocytes are considered to be suitable for *in vivo* assays (Rojas et al. 1992).

SCEs are very sensitive, although non-specific markers (Tucker and Preston 1996) efficiently induced by chemical substances that form covalent adducts to the DNA or otherwise interfere with DNA metabolism repair (Morimoto et al. 1985).

Results obtained by us showed a significant increase of SCE ($p < 0.05$; $p < 0.001$) in sheep peripheral lymphocytes after CCl_4 treatment in both assays i.e. with and without protective agent administration. However, comparison between groups of animals treated, hint at the effective influence of vitamin E and selenium on the level of SCE induced ($p < 0.01$). Tafazoli et al. (1998) pointed out in their report that mutagenicity of CCl_4 occurred in absence of significant cytotoxicity.

As our study showed, the administration of a single dose of 0.05ml/kg b.w. to sheep *in vivo* failed to exert of detectable cytotoxic effect in peripheral lymphocytes. Contrary to the positive influence of CCl_4 on SCE frequencies in the present study, neither of experimental groups did exhibit a significant decrease of proliferation indices.

Antioxidants such as vitamin E and selenium have a fundamental role in the prevention of oxidative damage. Vitamin E is present in the membrane components of the cell and prevents free radicals formation after CCl_4 influence (Fariss et al. 1993), while selenium functions throughout the cytoplasm to destroy peroxides mainly due to its involvement to the glutathione peroxidase activity (Ianas et al. 1995).

Under conditions of the present study, the simultaneous treatment of vitamin E and selenium with CCl_4 was confirmed. They could reduce the SCE frequencies in comparison to the non-protected individuals remarkably.

Genotoxicita indukovaná CCl_4 a protektívny účinok antioxidantov aplikovaných ovciam *in vivo*

V práci sme sledovali protektívny účinok injekčne podaných antioxidantov na genotoxické účinky indukované chloridom uhličitým. Desiatim ovciam veku 3 - 4 rokov bol aplikovaný chlorid uhličitý (CCl_4) orálne v dávke 0.05 ml/kg ž.h., ktorá bola odporúčaná v sedemdesiatych rokoch ako antihelmintická. Päť z týchto oviec však bolo 24 hod. pred podaním CCl_4 jednorázovo injekčne ošetrených vitamínom E (400mg) a selénom (12 mg). Ako kritérium pre hodnotenie genotoxických účinkov bola zvolená technika sesterských chromatidových výmen (SCE), ktoré sa hodnotili na 16. deň od aplikácie CCl_4 v kultivovaných lymfocytoch periférnej krvi. SCE, ktoré sa považujú za indikátory

replikačných porúch DNA, boli v obidvoch testovaných skupinách zvýšené, no v skupine protektívne ošetrenej vitamínom E a selénom sa táto hodnota v porovnaní so stavom pred pokusom zvýšila so štatistickou významnosťou $p < 0.05$, zatiaľ čo v skupine takto neošetrenej stúpla na hodnotu $p < 0.001$ (Studentov t– test), čo jednoznačne svedčí o protektívnom genotoxickom účinku podaných antioxidatív. Ani v jednej zo skupín nedošlo k štatisticky významnému poklesu proliferačného indexu, cytotoxický účinok na sledované tkanivo sa teda nedokázal.

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References

BELL, A., MEHENDALE, H. 1987: Comparative study in hepatic DNA, RNA, protein, lipid and glycogen induced by subtoxic dose of CCl_4 in Chlordecone, Mirex and Phenobarbital pre-treated rats. Toxicol. Lett**.** **35**: 191-200

BIASI, F., ALBANO, E., CHIARPOTTO, E., CORONGIU, F. P., PRONZATO, M. A., MARINARI, U. M., PAROLA, M., DIANZANI, M. U., PROLI, G. 1991: In vivo and in vitro evidence concerning the role of lipid peroxidation in the mechanism of hepatocyte death due to carbon tetrachloride. Cell Biochem. Funct. **9**: 111-118

CARRANO, A. V., NATARAJAN, A. T. 1988: Consideration for population monitoring using cytogenetic techniques. Mutat. Res. **204**: 397-406

CLAWSON, G. A. 1989: Mechanism of carbon tetrachloride hepatotoxicity. Pathol. Immunopathol. Res. **8**: 104-112

CREBELI, R., CARERE, A., LEOPARDI, P., CONTI, L., FASSIO, F., RAITERI, F., BARONE, D., CILIUTTI, P., CINELLI, S., VERICAT, J. A. 1999: Evaluation of 10 aliphatic halogenated hydrocarbons in the mouse bone marrow micronucleus test. Mutagenesis **14**: 207 -215

DA SILVA, A. L. G., LIEBER, S. R., RUIZ, M. A., DE SOUZA, C. A. 1997: Micronucleus monitoring to asses the human occupational exposure to organochlorides. Environ. Mol. Mutagen. **29**: 46-52

DIAZ GOMEZ, M. I., DE CASTRO, D. R., D` ACOSTA, N., DE FENOS, O. M., FERREYRA, E. C., CASTRO, J. A. 1975: Species differences in carbon tetrachloride – induced hepatotoxicity: the role of CCl_4 activation and lipid peroxidation. Toxicol. Appl. Pharmacol. **34**: 102-114

DOHERTY, A. T., ELLARD, S., PARRY, E. M., PARRY, J. M. 1996: An investigation into the activation and deactivation of chlorinated hydrocarbons to genotoxins in metabolically competent human cells. Mutagenesis **11**: 247-274

FARISS, M. W., BRYSON, K. F., HYLTON, E. E., LIPPMAN, H. R., STUBIN, C. H. ZHAO, X. G. 1993: Protection against carbon tetrachloride-induced hepatotoxicity by pretreating rats with the hemisuccinate ester of tocopherol and cholesterol. Environ. Health Perspect. **101**: 528-536

IANAS, O., OLINESCU, R., BADESCU, I., SIMIONESCU, L., POPOVICI, D. 1995: The influence of selenium organicum upon hepatic function of carbon tetrachloride poisoned rats. Rom. J. Intern. Med. **33**: 113-120

IKEGWUONU, F. I., MEHENDALE, H. M. 1991: Biochemical assessment of the genotoxicity of the in vivo interaction between chlordecone and carbon tetrachloride in rat hepatocytes. J. Appl. Toxicol. **11**: 303-310

LAMBERTI, L., PONZETTO, B. P., ARDITO, G. 1983: Cell kinetics and sister chromatid exchange frequency in human lymphocytes. Mutat. Res. **120**: 193-199

MILAD, K., KOVÁČ, G., BAJOVÁ, V., MOJŽIŠOVÁ, J. 2000: Effects of vitamin E and selenium on some metabolic and immunological parameters in sheep. Fol. Vet. **42**: 87-94

MORIMOTO, K., SATO–MIZUNO, M., KOIZUMI, A. 1985: Sister chromatid exchanges and cell-cycle kinetics in human lymphocyte cultures exposed to alkylating mutagens: apparent deformity in dose –response relationship. Mutat. Res. **152**: 187-196

NAROTSKY, M. G, KAVLOCK, R. J. 1995: A multidisciplinary approach to toxicological screening: II Developmental toxicity. J. Toxicol. Environ. Health **45**: 145-171

PAROLA, M., LEONARDUZZI, G., BIASI, F., ALBANO, E., BIOCCA, M. E., POLI, G., DIANZANI, M. U. 1992: Vitamin E dietary supplementation protects against carbon tetrachloride-induced chronic liver damage and cirrhosis. Hepatology **16**: 1014-1021

PERRY, P., WOLFF, S. 1974: New Giemsa methods for differential staining of sister chromatids. Nature **251**: 156-158

RABERGH, CH., LIPSKY, M. M. 1996: Toxicology of chloroform and carbon tetrachloride in primary cultures of rainbow trout hepatocytes. Aquat.Toxicol. **37**: 169-182

ROJAS, E., MONTERO, R., HERRERA, L. A., SORDO, M., GONSEBATT, M. E., RODRIGUEZ, R., OSTROSKY-WEGMAN, P. 1992: Are mitotic index and proliferation kinetics reproducible endpoints in genetic toxicity testing? Mutat. Res. **282**: 283-286

SUZUKI, H., HIRANO, N., WATANABE, C., TARUMOTO, Y. 1997: Carbon tetrachloride does not induce micronucleus in either mouse bone marrow or peripheral blood. Mutat. Res. **394**: 77-80

TAFAZOLI, M., BEATEN, A., GEERLINGS, P., KIRSCH-VOLDERS, M. 1998: In vitro mutagenicity and genotoxicity study of a number of short-chain chlorinated hydrocarbons using the micronucleus test and the alkaline single cell gel electrophoresis technique (Comet assay) in human lymphocytes: a structural activity relationship (QSAR) analysis of the genotoxic and cytotoxic potential. Mutagenesis **13**: 115-126

TAFAZOLI, M., KIRSCH-VOLDERS, M. 1996: In vitro mutagenicity and genotoxicity study of 1,2-dichlorethylene, 1,1,2- trichlotethane, 1-3 dichlorpropane, 1,1,3- trichloropropene, using the micronucleus test and the alkaline cell gel electrophoresis technique (comet assay) in human lymphocytes. Mutat. Res. **371**: 185-202

TUCKER, J. D., PRESTON, R. J. 1996: Chromosome aberrations, micronuclei, aneuploidy, sister chromatid exchanges and cancer risk assesment. Mutat. Res. **365**: 147-159

VAJDOVICH, P., SZILAGYI, A., GAAL, T. 1995: Evaluation of blood lipid peroxidation parameters in carbon tetrachloride CCl_4 in sheep. Acta Vet. Hung. **43**: 423-429

WAHLBERG, J. E. 1984: Erythema inducing effect of solvents following epicutaneus administration to man–studied by Doppler flowmetry. Scand. J. Work Environ. Health. **10**: 159-162

WEISBURGER, E. K. 1977: Carcinogenity studies on halogenated hydrocarbons. Environ. Health Perspect. **21**: 7-16

WESTBROOK-COLLINS, B., ALLEN, J. W., SHARIFF, Y., CAMPBELL, J. 1990: Further evidence that dichlormethane did not induce chromosome damage. J. Appl. Toxicol. **10**: 79-81