Effects of Fungicide Euparen Multi (Tolylfluanid) on Development of Preimplantation Embryos in Mouse

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Abstract

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The effect of the fungicide Euparen Multi (containing 50% tolylfluanid) on the development of mouse preimplantation embryos was evaluated. Euparen Multi was daily administered *per os* to female mice (ICR strain) at four different doses of 118, 294, 588 and 1177 mg/kg b.m., beginning on day 1 of pregnancy. Embryos obtained on day 4 of pregnancy were stained by morphological triple staining (Hoechst 33342, propidium iodide, Calcein AM), and the number of nuclei, blastocyst formation, distribution of embryos according to the nucleus number and cell death incidence were determined. Embryos in the experimental groups (except for the lowest dose 118 mg/kg b.m.) showed a highly significant dose-dependent reduction in total cell numbers corresponding to the lower proportion of blastocysts. The occurrence of cell death was significantly increased in all experimental groups, indicating that Euparen Multi is able to cause cell death at relatively low doses. Our data demonstrate that Euparen Multi could induce significant alterations in the preimplantation embryo development.

Tolylfluanid, fungicide, blastocyst, preimplantation embryo

At present many different pesticides and various pollutants are intensively introduced into the environment. People and animals are exposed to their influence. In many cases information about their toxic effects is not yet reliably confirmed. Although most of them entail increased risk, mainly for reproductive health, they are only now being more widely examined. Infertility (Greenlee et al. 2003) and congenital anomalies (Garry et al. 2002) are the usual adverse outcomes. Humans and animals usually come into contact with various foliage-applied agrochemicals. The main potential route of exposure for mammals is considered to be through the ingestion of residues in contaminated food items.

The commercial product Euparen Multi was chosen for this study because it is widely used in agriculture for controlling diseases in many fruit and vegetable crops. This product contains 50% of tolylfluanid [N-dichlorofluormethylthio-N'-N'-dimethyl-N-p-tolylsulphamide (IUPAC)] (TLF) as an active compound. It is a broad-spectrum phenylsulphamide fungicide, and is one of the most commonly detected pesticides registered in almost all member states of the EU. According to information from the producer and the results of several studies, TLF is generally regarded as a safe pesticide due to its short degradation time (1 - 2 weeks) after application in the environment (Logan 2002). It has been declared as non-genotoxic and non-carcinogenic (EFSA 2005). Toxicological studies carried out on laboratory animals have shown that technical-grade TLF is metabolized and eliminated from the organism in a very short time after its oral intake. TLF is of low toxicity in mice (LD_{50} > 1000 mg/kg b.m.) and rats (LD_{50} > 5000 mg/kg·b.m.); NOEC 100 mg/kg diet - chronic exposure 434 days) after oral administration

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(JMPR 2002 EC 2005). Health alterations were detected in mice and rats only after long-term oral application of TLF at high doses (750 mg/kg b.m.) accompanied by sedation, decreased motility and food intake and lower body mass (Logan 2002). Tolylfluanid has also been claimed to be non-teratogenic and relatively safe for reproductive health (1000 mg of TLF /kg had no visible effects on gestation and fertility, Logan 2002). While the effects of TLF on the postimplantation period seem to be non-significant, its influence on the preimplantation embryo development - a very sensitive part of the prenatal period - has not yet been described. Our aim therefore was to study the influence of short-term administration of different doses of this fungicide on the growth and development of mouse preimplantation embryos *in vivo*. In the presented experiment we used the commercial product Euparen Multi (50% TLF), because TLF is usually available and applied in the environment in this form.

Materials and Methods

Animals

Female mice (ICR strain, Velaz, Prague, Czech Republic; 4 - 5 weeks old) underwent superovulation treatment by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Bioveta, Ivanovice na Hané, Czech Republic), followed 46 h later by administration of 5 IU of human chorionic gonadotropin (hCG, Organon, Oss, Holland). Females were mated with males of the same strain overnight. Mating was confirmed by identification of a vaginal plug (day 1 of pregnancy). The animals were randomly allocated to four experimental groups and one control group. All animal experiments were approved by the Ethical Committee of the Institute of Animal Physiology SAS, Košice. The organization of the experiment, the investigations conducted, and the related documentation complied with legislative regulations governing the protection of experimental animals of the Slovak Republic.

Treatments

From day 1 to day 3 of pregnancy, females were daily administered by oral gavage (0.3 ml volume) four different doses of Euparen Multi (commercial product - Bayer, Leverkusen, Germany): group I - 118 mg/kg b.m. (approx. 6% of TLF LD_{50}), n = 30; group II - 294 mg/kg b.m. (approx. 15% of TLF LD_{50}), n = 30; group III - 588 mg/kg b.m. (approx. 30% of TLF LD_{50}), n = 43 and group IV - 1177 mg/kg b.m. (approx. 60% of TLF LD_{50}), n = 15, freshly prepared as a suspension in *aqua pro injectione*. The identical volume of *aqua pro injectione* was administered to control females.

Embryo recovery

Females were killed by cervical dislocation on day 4. Embryos were recovered at the blastocyst stage separately from each animal by flushing the uterus using a flushing-holding medium (FHM) (Lawits and Biggers 1993), and counted. The embryos were then transferred into 30 µl drops of FHM and prepared for morphological triple staining.

Morphological triple staining

For morphological analysis and cell death assessment, embryos were stained with cell-permeant dye Hoechst 33342 (HO, 20 µg/ml; Sigma-Aldrich; stains DNA of all cells), cell-impermeant dye propidium iodide (PI, 20 µ g/ml; Sigma-Aldrich; stains DNA of dead cells only), and Calcein AM (CA, 5 µM; BioChemika, stains cytoplasm of live cells only) for 40 min at 37 °C. The embryos were then washed, sealed with coverslips and observed using fluorescence microscopy at × 400 magnification (BX 51 Olympus, Japan).

The number of nuclei and corresponding morphological profiles were assessed in all embryos. According to their morphology (Hoechst), PI positivity/negativity and CA positivity/negativity, cells were classified into three groups (Fabian et al. 2004): normal cells (Hoechst normal PI - CA+; containing oval nuclei without morphological changes, able to exclude PI, with positive CA staining in cytoplasm); apoptotic cells (Hoechst damaged PI ± CA+; containing nuclei with typical fragmented or condensed morphology, PI negative in early stages, PI positive in the final stage of apoptotic process, usually with positive CA staining in cytoplasm); necrotic cells (Hoechst normal/damaged PI + CA-; mostly without specific morphological changes, always PI positive and CA negative).

Occasional mitotic configurations were also classified as normal nuclei (Fabian et al. 2004). The percentage of dead cells represents the proportion of both apoptotic and necrotic cells relative to total embryo cell number.

The following numbers of embryos were examined by morphological triple staining on day 4: group I - Euparen Multi 118 mg/kg b.m., n = 262; group II - 294 mg/kg b.m., n = 328; group III - 588 mg/kg b.m., n = 411; group IV - 1177 mg/kg b.m., n = 103; control group n = 1194.

Statistical analysis

The results are expressed as mean values ± S.D. Chi-square test was used to detect differences in the formation of blastocysts, distribution of preimplantation embryos according to their total cell number and cell death incidence.

The one-way ANOVA followed by Dunnett's test were used for the statistical analysis of total cell numbers of embryos. Values of $P < 0.05$ were considered as significant.

Results

The analysis of embryo growth and development influenced by Euparen Multi evaluated by triple staining is shown in Table 1.

Table 1. Analysis of development and cell death incidence in mouse preimplantation embryos obtained on day 4 from ICR mice daily administered "Euparen Multi" (containing 50% tolylfluanid) beginning on day 1 of pregnancy, evaluated by morphological triple staining (Hoechst, PI, Calcein AM)

	Control	Group I (118 mg/kg b.m.)	Group II (294 mg/kg b.m.)	Group III (588 mg/kg b.m.)	Group IV (1177 mg/kg b.m.)
No. of pregnant mice	139	30	30	43	15
No. of retrieved embryos	1194	262	328	411	103
Mean No. of nuclei/embryo	53.6 ± 12.7	55.3 ± 17.2	44.0 ± 14.6***	44.1 ± 15.5***	36.3 ± 16.2***
Percentage of blastocysts with > 33 nuclei	87.8	87.0	74.4***	70.8***	48.5***
Percentage of embryos with					
1 - 8 nuclei	2.5	4.2	1.5	5.1	10.7
9 - 16 nuclei	1.9	1.5	3.1	2.9	12.6
17 – 32 nuclei	7.9	7.3	21.0	21.2	28.2
33 – 64 nuclei	63.1	60.7	65.5	62.3	46.6
> 65 nuclei	24.0	26.3	9.2***	8.5***	1.9***
Percentage of normal cells in embryos	99.0	98.7	98.2	98.0	96.1
Percentage of dead cells in embryos	1.0	1.3**	1.8***	2.0***	3.9***

Negative effects of Euparen Multi on mouse preimplantation embryos appeared in all studied parameters and in all treated groups with progressive intensity in a dose-dependent manner. In embryos obtained from mice treated with Euparen Multi the number of nuclei was significantly lower ($P < 0.001$) in comparison to the control, except for the group treated with the lowest dose of 118 mg/kg b.m. (group I - 55.3 ± 17.2, control 53.6 ± 12.7), where the differences were non-significant.

The distribution of embryos according to nucleus number was characterized by a progressive decrease in Euparen Multi-treated groups, with the lowest cell numbers at the highest dose of 1177 mg/kg b.m. ($P < 0.001$).

The number of blastocysts was closely linked to the previous two parameters. The blastocyst formation was comparable to controls only at the dose of 118 mg/kg b.m. - (group I - 87.0%, control 87.8%). On the other hand, the lowest blastocyst formation (48.5%) was detected in the group treated with 1177 mg/kg b.m. ($P < 0.001$). Highly significant differences ($P < 0.001$) were also found in the two groups treated with 294 mg/kg b.m. (74.4%) and 588 mg/kg b.m. (70.8%) . Highly significant differences ($P < 0.001$) in all four Euparen Multi-treated groups in comparison to the control group were found in the rate of embryo cell death (apoptosis or necrosis) and the proportion of death cells increased progressively with the dose used. The highest number of dead cells was found at the dose of 1177 mg/kg b.m. (group IV - 3.9%, control 1.0%).

Discussion

Pesticides and other pollutants can alter prenatal development in both pre- and postimplantation periods. Overall effects of pesticides on the mammalian embryo

development are precisely documented mainly during the postimplantation period. Available data on pesticide effects on the preimplantation embryo development in mammals seem to be limited, although this period is presented as the most sensitive, and altering effects occurring during this phase may have serious consequences for later development. Mainly organochlorine pesticides have been studied from this point of view (Alm et al. 1996; Campagna et al. 2001).

Amstislavsky et al. (2003) reported alterations in mouse preimplantation embryos caused by the pesticide methoxychlor administered to pregnant mice at the dose of 16.5 mg/kg of b.m. during days 2 - 4 of pregnancy. In a later study it was shown that alterations induced by *in vivo* metoxychlor exposure led to a high mortality rate in later phases of embryonal development, and altered postnatal sexual development, especially in male offspring (Amstislavsky et al. 2004). Similarly, the presented results indicate that the short-term peroral intake of Euparen Multi could negatively influence the preimplantation embryo development, even though it has been characterized as relatively safe. We show that embryos isolated from experimental females had a decrease in cell proliferation leading to impaired growth and a reduction in blastocyst formation.

We observed highly significant differences induced by Euparen Multi treatment in comparison to the control group even at the dose of 294 mg/kg b.m. (corresponding to 147 mg TLF/kg b.m.), and higher doses of this fungicide induced more profound changes. To our knowledge, there are no available data regarding the effects of TLF on the preimplantation embryo development in the mouse. Similar dose response effects were reported by Greenlee et al. (1999) after 96 h cultivation of pronuclear (2PN) embryos with addition of o,p-DDT, which significantly reduced embryo development to the blastocyst stage as well as mean cell number. These authors also noted an increase in the percentage of cells undergoing apoptosis. A single dose (40 or 80 mg/kg) of chlorpyrifos (an organophosphate pesticide) administered i.p. to pregnant female mice induced cytogenetic damage in preimplantation embryos and there was also a significant decrease in the embryo cell number in the 80 mg/kg treated group (Tian and Yamauchi 2003). Scascitelli and Pacchierotti (2003) tested lindane (γ-isomer of hexachlorocyclohexane) for developmental alterations during early embryonic cleavage using daily doses of 15 or 25 mg/kg b.w. Two-cell embryos and morulae exhibited lysis or fragmentation of blastomeres and cell proliferation delay. However, a statistically significant increase in degeneration of two-cell embryos was induced only by the higher dose of lindane. The incidence of cell death caused by Euparen Multi was within the range of 1.3% to 3.9% and was presented mostly as necrosis. The increase of the proportion of death cells was highly significant and was detected at all four doses in a dose-dependent manner. Despite the apparently low levels of embryo cell death in our experiment, they could be of physiological significance due to a generally low proportion of death cells in *in vivo* developed blastocysts (Brison and Schultz 1997). Greenlee et al. (2004) reported a considerably higher percentage of death cells when they evaluated the effect of two fungicides - chlorothalonil (12.54%) and mancozeb (13.62%) - on mouse preimplantation embryos. Cell death was presented as apoptosis and no necrotic lesions were observed.

The health status of females was visibly negatively affected in the experimental group fed the highest dose of Euparen Multi (1177 mg/kg b.m.), and in fact one third of animals died during the experiment. Inflammation of the digestive tract, observed during the experiment could be the main cause of death. Apparent differences between the results of this study and available literary sources could be explained to a certain extent by different experimental methods - different animals (mice/rat), mode of pesticide administration (our experiment - short term by oral gavage, others - medium to long term addition to the diet), purity of TLF. The study carried out on pregnant rats showed no deaths or behavioral changes and no

biologically or statistically significant effects on gestation and fertility indices after application of high dose of technical grade TLF (purity 99.9%) at 1000 mg/kg b.m. per day (Logan 2002). The only clinical sign observed was hair loss and decreased body mass. Similarly, another study of reproductive and developmental toxicity in rats showed that only high doses of TLF caused restricted body mass gain observed in dams at 300 and 1000 mg/kg b.m. per day. Reduced fetal body mass and an increased resorption rate were seen at 1000 mg/kg b.m. per day applied in the postimplantation stage. The reproductive toxicity was shown in a multigeneration study of pregnant female rats treated with TLF, and the dose 7.9 mg/kg b.m. per day was established as NOAEL (Logan 2002).

Several persistent environmental pollutants have been reported to interfere with steroid receptors belonging to the nuclear receptor family. Johansson et al. (2005) examined the affinity of tolylfluanid to the glucocorticoid receptor extracted from mouse liver cytosol and they have shown antagonistic effects of tolylfluanid on the glucocorticoid signal transduction and TLF affinity to the mouse glucocorticoid receptor. It could be hypothesized that the observed negative effects of tolylfluanid on the preimplantation embryo development in our experiment were related to tolylfluanid interactions with glucocorticoid receptors.

Results of the present study indicate that Euparen Multi is able to cause significant alterations of embryo development and decrease the viability of preimplantation embryos. These changes were detectable already at low doses without any apparent changes in the health status of laboratory animals. We hypothesize that Euparen Multi may present a certain risk for the reproductive health of animals and possibly also for human reproduction. The mouse preimplantation embryo assay appears to be a sensitive and reliable way of assessing early developmental injury due to Euparen Multi short-term exposure at doses below those producing visible health effects. Unfortunately, experiments with *in vitro* preimplantation embryo culture are difficult to accomplish due to the low solubility and rapid degradation of TLF. Short-term *in vivo* exposure of murine preimplantation embryos to experimental treatment provided reproducible comparison of pesticide effects on developmental outcome (blastocyst development, embryo cell number and proportion of cell death). However, additional work is necessary to estimate possible human risk and to determine the relevance of early *in vivo* exposure to Euparen Multi for the outcome of pregnancy.

Vplyv fungicídu Euparen Multi (tolylfluanid) na vývin preimplantačných embryí u myší

Študovali sme vplyv fungicídu Euparen Multi (obsahujúci 50% tolylfluanidu) na vývin preimplantačných embryí u myší. Euparen Multi bol denne perorálne podávaný samičkám myší (kmeň ICR) od prvého dňa gravidity v štyroch rôznych dávkach 118, 294, 588 a 1177 mg/kg ž.h.. Embryá získané na 4. deň gravidity boli analyzované pomocou morfologického trojitého farbenia (Hoechst 33342, propídium jodid, Calcein AM) a bol zistený počet jadier, tvorba blastocýst, distribúcia embryí vzhľadom na počet jadier a výskyt bunkovej smrti. Embryá v experimentálnych skupinách (okrem najnižšej dávky 118 mg/kg ž.h.) v závislosti na použitej dávke mali vysoko preukazne znížený celkový počet buniek čo sa prejavilo v zníženom zastúpení blastocýst. Výskyt bunkovej smrti bol signifikantne zvýšený vo všetkých experimentálnych skupinách, čo naznačuje, že Euparen Multi môže spôsobiť smrť buniek v relatívne nízkych dávkach. Naše výsledky svedčia o tom, že Euparen Multi môže vyvolať preukazné zmeny vo vývine preimplantačných embryí.

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