Bull. Environ. Contam. Toxicol. (2001) 67:821–827

DOI: 10.1007/s00128-001-0196-6

Bulletin of Environmental Contamination and Toxicology

Effect of Endosulfan on the Enzymes of Polyol Pathway in Rat Sertoli-Germ Cell Coculture

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Received: 1 October 2000/Accepted: 6 August 2001

Culture systems are being used to investigate the mode of action of toxicants, once testicular toxicity has been observed *in vivo*. Endosulfan (6,7,8,9,10-hexachloro, 1,5,5a,6,9,9a - hexahydro, 6-9-methano-2,3,4 benzodioxa-thiepin-3-oxide)an organochlorine insecticide is used extensively in agriculture to control pests of vegetables, cotton, fruits etc.in India and elsewhere (Toxicological Profile, 1993). The residual accumulation of endosulfan has been reported in various vegitables of India and other developing countries (Dushra et.al. 1984; Dethe et.al. 1990). The absorption of endosulfan in the body is either through ingestion, inhalation and skin contact. *In vivo* studies have indicated that endosulfan leads to testicular impairment both in adult and young growing rats (Sinha et. al. 1995, 1997). No work is reported till date on the mechanism of endosulfan induced testicular toxicity employing testicular cells in culture. Recently, we have shown the cytotoxic effect of endosulfan on rat Sertoli-germ cell coculture which was evident by germ cell detachment, loss of viability and leakage of cytosolic enzyme lactate dehydrogenase in a dose dependent manner (Sinha et.al. 1999). This effect may be due to the disruption of certain metabolic pathways existing between Sertoli cell and germ cell which is necessary for maintaining integrity of germinal epithelium. The polyol pathway is one such metabolic co-operation existing between Sertoli cells and germ cells (Foster, 1988). In the present work, we report the effect of endosulfan on the enzymes of polyol pathway namely sorbitol dehydrogenase and aldose reductase which are located specifically in germ cells and Sertoli cells respectively,in rat Sertoli-germ cell coculture. The normal functioning of these enzymes is necessary for the proper interaction occurring between the two cells.

MATERIALS AND METHODS

Eagle's minimum essential medium (EMEM), Collagenase

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(type I), DNase I were procured from Sigma, St. Louis, Mo. Hank's balanced salt solution (HBSS), fetal calf serum (FCS) and trypsin were purchased from Hi-media laboratory Pvt. Ltd., and NADH, NADPH were procured from Sisco Research Laboratories Pvt Ltd. Mumbai, India. Tissue culture dishes were obtained from Tarson, India.Technical grade endosulfan (95.32% purity) was a gift from M/S Bharat Pulverising Mills Pvt. Ltd. Mumbai, India.

Weaned male albino rats of Druckray strain were procured from the Industrial Toxicology Research Centre animal breeding colony and housed in polycarbonate cages in an air conditioned room with 12-h light/dark cycles and 60-70% humidity. Animals were acclimatized for a week prior to experimentation and fed a pelleted diet (Ashirwad, Ropar, Punjab, India) and water ad libitum.

Mixed cultures of Sertoli and germ cells was prepared according to the methods of Ku and Chapin (1988) with slight modification already mentioned elsewhere (Sinha et.al. 1999). In brief, testis from 28-30d old rats were collected in sterile HBSS under asceptic condition. Following decapsulation, the testis were minced in sterile HBSS and digested with trypsin (0.4%) followed by collagenase (0.1%) for 15 and 45 minutes at 32ˆC respectively. The flask was manually shaken during the process of digestion. It was then washed with EMEM (containing 100 ug penicillin/ml. 100ug streptomycin/ml) containg 10% FCS. The centrifuged pellet was subjected to repeated washing with EMEM until the supernatant was clear. The pellet served as the Sertoli-germ cell coculture stock. Aliqotes (3.5 ml) of this suspension were plated in 35 mm tissue culture dishes and incubated at 32ˆC in a humidified atmosphere with 5% CO_2/95% air. The cells were plated at a density of $11x10^6$ cells/dish (Sinha et.al.1999).

After preincubation for 24 h (as mentioned above) the medium was replaced with fresh serum free medium containing endosulfan (2, 20, 40 and 80 uM) dissolved in dimethyl sulphoxide (DMSO; final DMSO concentration 0.4% v/v) (Sinha et.al. 1999). Control cultures received medium containg 0.4% (v/v) DMSO only. The cultures were then incubated for 48 hours (a total of 72 hrs), the medium being replaced after every 24 h with fresh medium containing same quantity of test material.

Following 24 and 48 h treatment, the medium was discarded by decanting and the adherent cells were collected by slight aspiration/and or scrapping with fresh EMEM. The process was repeated unless the whole of adherent cells were collected. The cells were centrifuged at 800 rpm and pellet collected was further washed with sodium phosphate buffer (pH 6.4). The pellet obtained

was homogenised in sodium phosphate buffer and centrifuged at 10,000 rpm to collect post-mitochondrial fraction. The supernatant collected was used as the source of enzyme.

The enzyme aldose reductase (ARD) and sorbitol dehydrogenase (SDH) was estimated according to the methods of Tanimoto et.al. (1983). In brief, aldose reductase activity was determined by measuring the decrease in absorption of NADPH at 340nm in an spectronic-2000 spectrophotometer (Bausch and Lomb). Sorbitol dehydrogenase activity was determined in the same manner as aldose reductase activity except that D-fructose was used as the substrate (whereas D-glucose in case of aldose reductase) and NADH as the co-enzyme. One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation of 1umol of NAD(P)H per minute under the assay condition described above. Protein in the sample was also measured (Lowry et.al., 1951).

Data were analysed with Student's t-test. The significance level was ascertained at $P<0.05$.

RESULTS AND DISCUSSION

Mixed cultures of Sertoli and germ cells from 28-d old rats generally contains Sertoli cells, primary spermatocytes at various satages of meiotic division and few spermatogonia.

Sertoli cell monolayer was obtained after 20-24 hr of initial plating with attached germ cells (cell were 92-95% viable as assessed by the trypan blue exclusion test (Sinha et.al., 1999).

The activity of aldose reductase was calculated as nmol NADPH oxidised /min/mg protein. Results revealed an increase in the activity of the enzyme aldose reductase in Sertoli-germ cell coculture exposed to 20 - 80 uM endosulfan as compared to control and 2uM dosed group both after 24 and 48 hrs treatment (table 1). The pattern of increase was in a dose dependent manner. Duration dependency was observed only in the higher dosed groups (40-80 uM).

The activity of sorbitol dehydrogenase was calculated as nmol NADH oxidised/min/mg protein. A dose dependent decrease in the activity of this enzyme was recorded from 20 to 80uM exposed group after 24 hrs of endosulfan treatment (table 2). However, the enzyme activity recorded from culture exposed to 2uM of endosulfan was similar to that of control. The trend of the activity was similar when the exposure time was further increased to 48 hrs i.e dose dependent increase

in the activity in 20 to 80 uM endosulfan exposed groups. However, no duration dependent changes in this enzyme activity was observed as compared to 24 hours treatment.

Table 1. Effect of endosulfan on aldose reductase (ARD) in rat Sertoli-germ cell coculture

	ARD (nmol NADPH oxidised/min/mg protein)	
Endosulfan conc.	Treatment time (h)	
(uM)	24h	48h
0	1.56 ± 0.03	1.55 ± 0.03
2.0	1.54 ± 0.03	1.56 ± 0.01
20.0	1.80 ± 0.02^{ab}	1.79 ± 0.02^{ab}
40.0	2.50 ± 0.10^{abc}	2.96 ± 0.01^{abc*}
80.0	3.20 ± 0.10^{abcd}	3.89 ± 0.05^{abcd*}

Table 2. Effect of endosulfan on sorbitol dehydrogenase (SDH) in rat Sertoli-germ cell coculture.

	SDH (nmol NADH oxidised/min/mg protein)	
Endosulfan conc.	Treatment time (h)	
(uM)	24h	48h
0	7.89 ± 0.26	7.18 ± 0.22
2.0	7.49 ± 0.14	6.80 ± 0.24
20.0	6.09 ± 0.19^{ab}	5.28 ± 0.25^{ab}
40.0	4.87 ± 0.10^{abc}	4.43 ± 0.13^{abc}
80.0	3.79 ± 0.15^{abcd}	3.21 ± 0.19^{abcd}

Values are mean ± SE three culture dishes per concentration. $P<0.05$ by Student's t-test compared to: a- Control; b- 2 uM endosulfan; c- 20 uM endosulfan; d- 40 uM endosulfan. *- Corresponding 24 hrs value

Sertoli cell-germ cell interaction is generally considered to be required for the differentiation of male germ cells and their progression through the seminiferous epithelium upto the release of mature

spermatozoon, in the tubular lumen (Russel, 1977; Sundaran and Witorsch, 1995). Some of the interesting aspects of Sertoli-germ cell interactions have focussed on the metabolic cooperation between Sertoli cells and spermatocytes and/or spermatid (Foster, 1988). Polyol pathway is one such metabolic co-operation existing between Sertoli and germ cells which is necessary for maintaining integrity of germinal epithelium. Ludvigson et.al. (1982) have elegantly shown using immunohistochemistry that the enzymes aldose reductase is specifically located in Sertoli cells. This enzyme is responsible for the reduction of aldoses to polyols (eg glucose to sorbitol). These polyols are in turn taken up by the germ cells and converted to fructose (and other ketoses) by the enzyme, sorbitol dehydrogenase (Foster, 1988). In the present study, an increase in the activity of aldose reductase and decrease in the activity of sorbitol dehydrogenase in Sertoli-germ cell coculture at certain doses of endosulfan exposure reflects the interference of this pesticide in the process of metabolic cooperation existing between Sertoli and germ cells. The effect was in a dose dependent manner. Fukuoka et.al (1989) have also shown that the activity of aldose reductase increases with the progress of testicular atrophy. They have further suggested that this may result probably due to a change in the metabolism of glucose to inositol which is reported to be a metabolic requirement for the normal progression of spermatogenesis (Morris and Collins, 1971). Sertoli cells have the higher levels of the enzymes required for inositol biosynthesis from glucose, while in spermatocytes & spermatids this enzyme remains non detectable (Venkatachalam et.al., 1983).

The enzyme sorbitol dehydrogenase which is also a marker of germ cell number, has been shown to increase with the maturation of testis *in vivo* (Hodgen and Sherins, 1973). This is because of enhanced fructose production for energy requirement by the testis.A decrease in the activity of sorbitol dehydrogenase in the present study suggests an impairment in the process of conversion of sorbitol to fructose. This result is further supported by our earlier report where we have shown germ cell exfoliation at the same doses of endosulfan exposure both after 24 and 48 hours of treatment. Fakuoka et.al (1989) have shown that Di-n-butyl pthalate induced testicular atrophy can be possibly due to the disruption of polyol pathway.

Thus, this study suggests that endosulfan disrupts the polyol pathway by affecting the specific enzymes responsible for the same which may be one of the reasons leading to cytotoxicity *in vitro*. This is one of the possible metabolic interactions between Sertoli cells and germ cells which toxicologists are now investigating

for potential uses as indices of Sertoli-germ cell function and viability (Foster 1988).

Acknowledgments We thank the Director of Industrial Toxicology Research Centre for his keen interest and encouragement in this study. The authors, NS and NA, are thankful to Council of Scientific and Industrial Research, New Delhi, for providing financial assisstace as Senior Research Associateships. We thank MS Bharat Pulverising Mills Pvt. Ltd. Mumbai for supply of technical grade endosulfan as a gift. ITRC COMMUNICATION NO.: 2065

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