Expression of vimentin protein and neurofilament on forelimb buds of black-6 mice on gestation day 12 induced by 2-methoxyethanol by RT-PCR

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Abstract. Irnidayanti Y. 2010. Expression of vimentin protein and neurofilament on forelimb buds of black-6 mice on gestation day 12 induced by 2-methoxyethanol by Real Time RT-PCR. Nusantara Bioscience 2: 116-120. The aim of this study was to investigate impact of 2-methoxyethanol, a major industrial chemical of plastic. Gene expression analysis is increasingly important in biological research, while real-time reverse transcription PCR (RT-PCR) is becoming the method of choice for high-throughput and accurate expression profiling of selected genes. Pregnant black-6 mice were injected intraperitoneally to 7.5 mmol/kg of 2-methoxyethanol on gestation day (GD) 10. Embryo were obtained on gestation day 12. Forelimb buds of embryo was collected and then put in the tube, which containing RNA-latter solution. To identify gene expression changes in forelimb bud caused induction 2-methoxyethanol, Real Time PCR were using in this research. For the experiments the real-time RT-PCR Light Cycler technology was used. The results suggested that injection of 2-methoxyethanol, in prenatal period especially on gestation day 12, the expression of vimentin in forelimb buds of mice treatment increase than control mice. Meanwhile the expression of neurofilament tended to decrease, indirectly is not caused by the injection of 2-methoxyethanol.

Key words: vimentin, neurofilament, 2-methoxyethanol, limb bud, black-6 mice.

INTRODUCTION

The compound of 2-methoxyethanol (2-ME) or ethyleneglycol methyl ether is one of the glycol ether compounds derived from the compounds of phthalate esters. This compound is widely used as the basic material of plastic. Plastic is very useful in everyday life. People use plastics in their everyday life. Plastic is generally used for a variety of human activities, such as household appliances, packing materials, bottles, food containers, toys, water pipes and even used for the purposes of health such as blood storage for transfusion.

The waste of the compounds is often wasted in the environment and being the cause of pollutants, particularly in the aquatic environment or the river (Miller et al. 1983). The compounds were known to be toxic or teratogenic in several mammalian species (Feuston et al. 1990). Some previous reports also mentioned that, some people have been poisoned with 2-ME through penetration into the skin and bronchial tube (Dugard et al. 1984). Approximately 100,000 people were poisoned by 2-ME per year, of which allegedly were women who were still in the fertile period or able to give birth (Scott et al. 1989). The teratogenic of 2-ME in experment animal is caused by the metabolism of 2-ME in the hepatic cells transforming into methoxyacetic acid (MAA), by the help of a catalyst namely alcohol dehydrogenase (Brown et al. 1984; Moslen et al. 1995).

Our previous studies mentioned that, 2-ME causes abnormalities in fetal mice whose dams was given 2-ME or...
MAA, whose main disorders that appear were abnormalities the skeletons, the disorder on the axial skeleton, due to the damage of embryonic somit tissues which in turn led disorders on the spines and ribs, spina bifida; and exencephaly (Darmanto et al. 1994; Darmanto 1998). Some researchers have reported that after giving MAA with a single dose of 10 mmol/kg body weight on gestation day 11 of in mice JCL: ICR (Rasjad et al. 1991), AJ (Sudarwati 1993), Swiss Webster, (Suripto et al. 1996), showed that 94% of limb bud of the fetus experiment was abnormalities.

The nature of both Embryo toxic and teratogenic MAA compound has been shown in mammals, especially in embryos both pre and post implantation periods. This embryo toxic characteristic is similar to that caused by the compound 2-ME (Darmanto et al. 1994).

Limb bud is a good model to study the pattern of certain growth and also to understand the possibility of developmental disorders caused by specific teratogens (Ruyani et al. 2008). The results of the research conducted by Rasjad et al (1991), shows that the distribution pattern of defects caused by MAA, a metabolite of 2-methoxyethanol, is caused by the difference in the number and distribution of dead cells that occurs in the mesoderm of the limb plate. The result of microscopic observations showed that signs of limb bud abnormality that experienced necrosis of mesenchymal cells and the AER (apical ectoderm Ridge), which was observed for 2 hours after being given MAA at gestation days to 10.5, then after 6 hours showed hyperplasia on AER. AER itself plays a role in the formation mechanism of the disorder, because there is a degenerative change in structure and a more rapid depreciation in the fetus that was being treated compared than control (Sudarwati, 1995). The results of the research by Mebus and Welsch (1989) that MAA treatment may interfere with the availability of purine and pyrimidine bases, which are expected to affect DNA and/or RNA synthesis, which in turn influence normal cellular proliferation and differentiation in the developing mouse embryos.

In the process of formation of a normal limb bud, there are several phases: proliferation, migration, differentiation and cell death phase. When the embryo is gestation days 10, it is an early stage of initial bud formation, in which cells undergo proliferation by forming AER.

In addition, there is also migration of miogenic cells on the buds. The muscular structure of the buds is partly from the miotom somites. The migration of miogenic cells begins soon after the formation of buds. The bud cells have the ability to aggregate forming two entities of muscles namely dorsal muscles and premuscular muscles, which are formed at the early stages of miogenic differentiation (Ewan and Everett, 1997). Miogenic cells accumulated in the buds expressed vimentin proteins (Hayasi et al. 1993). The Results from Vaittinen et al. (2010), indicate that vimentin proteins play roles in miogenesis, both functional roles in the construction and restoration of skeletal muscle fibers. The presence of vimentin proteins related with cellular function of cells during embryonic development. So we can say that vimentin is expressed in the in early phase of organogenesis, which is characterized by the aggregation and condensation of prekartilago (Viebahn et al. 1988). Vimentin role tansports kinase into the cell nucleus. The kinase activates the neurons and affect gene expression in such a way that neurons can respond to the damage. This proves that vimentin is required in the repair of scar tissue through the migration of cells and tissue formation (Moon et al. 2004).

The expansion of those aggregated cells will stop when it reaches the form of condensation of pre-cartilages. The form of pre-cartilages condensation will trigger cartilage differentiation, which eventually forms cartilage (Lee et al. 1998). This development process includes the activities of cells namely cell migration, cell adhesion, intracellular signaling and cell proliferation. The process of proliferation and migration of these miogenic cells are influenced by fibronectin. This protein helps facilitate the migration of miogenic cells, as a place of attachment and the guidance of the migration.

Neurofilament are a group of proteins that guide cells during the organogenesis process, if the expression level of these compounds are disturbed by the compound 2-ME, it is assumed that there will be abnormalities of limb bud. However, the abnormality that occurs is in the form of polydactyl, caused by dead cell, which is not the only mechanism that causes the appearance of disorder. Another research states that a failure in the functioning of the longitudinal and circular smooth muscle in cases of stenosis pyrolus, is known associated with the unexpressed ncam and neurofilamen or indicating the absence of innervations on the smooth muscle tissues (Kobayashi et al. 1995). The development of the embryo limb buds would be disrupted, with a decrease of the expression neurofilamen protein (Nicholas et al. 2003). Based on these results there might be any of a certain kind of disturbed protein expression that was induced by 2-ME compound. In this study, it is invetigated the expressions of these proteins induced by 2-ME compound in the critical period of the development of the buds, in real time RT-PCR. It is expected to know the proteins that are expressed during the formation of bud members and it is also expected to reveal the role of proteins in causing abnormalities limb buds.

**MATERIALS AND METHODS**

**Experimented animals**

Black-6 mice were used in this study were brought from Charite-Universutats Medizin Berlin. vaginal plug detected the following morning was defined as day 0 of gestation (Rugh, 1968). The pregnant mice were killed by cervical dislocation at gestation days 10 (GD-10). The sample of the buds members were isolated under the microscope, and then put into the tube containing the RNA-latter, for the analysis of RNA and DNA. Test RNA was conducted with RNeasy Kits Real Time RT-PCR.

**Materials, dosage and samples collection**

2-ME in liquid form (Product Number: 135–07762) was produced by Wako Pure Chemical Industries, Ltd. Japan.
2-ME diluted with sterilized distilled water was administered by peritoneally injection at a dose of 7.5 mmol/kg body weight on GD-10. Control mice were given sterile distilled water with the same dose. On the GD-12, the mice were killed by cervical dislocation, and then the fore limb buds were isolated and put in tube with the latter RNA solution, then stored for further analysis at a temperature of -20°C.

**Real Time RT-PCR**

The total RNA forelimb buds tissue was extracted with the RNeasy kit according to the manufacturer’s protocols. cDNA was synthesized from the total RNA using the Qiagen One Step RT-PCR Kit (Cat. No. 210 210). PCR reactions using enzymes AidTM H Minus M-MuLV RT (Cat. No. 130 125 486) at a temperature of 95°C, 7 min, 45 cycles of PCR (20 sec, 95°C, 60°C, 20 sec, 72°C, 30 sec), 42°C, during 1 hour 15 minutes, 70°C elongation then followed with the temperature of 70°C, for 5 minutes. Quantitative analysis performed by Real-Time PCR. Analysis of Polymerase chain reaction (PCR) was done by adding each cDNA 9.00 µl of treatment or control forelimb buds and 1 µl of Primary-Mix into each different tube. In our experiments, Primary-Mix consists of 4 primary types of vimentin and NFh, Nfm, and the NFl. In buds of both the control and treated, then was added by the component of reactions that was aqua, SYBER Green, 2x Bioline buffer. Then reaction of Real Time RT-PCR showed the complete series of targeted cDNA, followed by Oligonucleotide primers. The Primers used in this study were synthesized into Biotez Berlin-Buch GmbH, Berlin, Germany. Information on primers can be seen in Table 1.

**Table 1. Sequencing orimer position (f = forward; r = reverse), % contents of G/C dan referency primer product**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>GC (%)</th>
<th>TIB reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vim-f</td>
<td>CTG AGG CTG CCA</td>
<td>59,1</td>
<td>011701.3:1376F22</td>
</tr>
<tr>
<td>Vim-r</td>
<td>CCT CGC CTT CCA</td>
<td>66,7</td>
<td>011701.3:1682R21</td>
</tr>
<tr>
<td>Nef h-f</td>
<td>AGG AGA TAA CTG</td>
<td>66,7</td>
<td>010904.3:1349R23</td>
</tr>
<tr>
<td>Nef h-r</td>
<td>CGA GCA CAC TCT</td>
<td>59,1</td>
<td>008691.2:1055F22</td>
</tr>
<tr>
<td>Nef m-f</td>
<td>GTG GTT CAA ATG</td>
<td>66,7</td>
<td>008691.2:1432R21</td>
</tr>
<tr>
<td>Nef m-r</td>
<td>GAG GCC CGG TGA</td>
<td>52,2</td>
<td>010910:1221F23</td>
</tr>
<tr>
<td>Nef l-f</td>
<td>TGG CCT TGG ACA</td>
<td>52,2</td>
<td>010910:1489R21</td>
</tr>
<tr>
<td>Nef l-r</td>
<td>GCT CTC TCA CAG</td>
<td>66,7</td>
<td>010910:1489R21</td>
</tr>
</tbody>
</table>

**Table 2. DNA cycle conditions forelimb buds proteins UK-12-day embryo induced by 2-ME in real-time reverse transcription-polymerase chain reaction (RT-PCR).**
In Table 2, it is shown that the number of copies of genes for vimentin expression is very high compared with vimentin protein gene in the control group. The number of the copies of genes is around 272,989, relatively higher compared than control that is 37,127. While the number of the copies of neurofilamen low protein in the treatment group was lower than the control group, that is 38,269 for the group of treatment and 47,927 for that of control. While the medium and high neurofilament are very low in the treatment group that are 9416 and 849, while in the control are 2327 and 200.

RESULTS AND DISCUSSION

Results of DNA copies by the real time RT-PCR is from the mice black -6 using the primers of vimentin and high, medium and low neurofilamen low (figure 1). In the picture above, it is shown that the expression of vimentin in the early development of limb buds is very high. Other proteins such as nf-low look the opposite that is relatively decrease, when compared with the control group.

The limb buds firstly appear on the 10th day of gestation, which seem a group of mesenchyme cells that grow like forelimb bud. Mesenchyme cells that cover the primordium limb buds will form the cube-shaped cells derived from ectoderm layer. This form is known as the apical ectoderm Ridge (AER). On the 12th gestation day, the limb buds turn themselves in the shape of polygonal or rather pentagonal. The role of proteins in the development of limb buds is very high. Other proteins such as vimentin genes for vimentin expression is very high compared with control one. Under normal circumstances, the expression of vimentin protein in embryonic period on GD-10 is very high (Iridayanti, 2009), then gets decreased on the GD-12. But in this study, after injected with 2-ME, the expression of vimentin protein appears the opposite, that it gets increased on GD-12. These data are supported by Vaittinen et al (2001), that the expression of vimentin increases maximum for 3-5 days after the post injury on the mioblas. This means that after being given with 2-ME, there occurs damage on limb buds tissue. In the damaged tissue, it can be seen the raising of the expression of vimentin protein. The high expression of vimentin protein is needed to repair the damaged tissue induced by 2-ME.

Based on the research by (Viebahn, 1988) showed that the existence of protein expression appear to be associated with cellular functions during embryonic development. Vimentin, which is an intermediate filament, acts as transduction signal (Helfand et al. 2005). Vimentin can interact with the MAP (mitogen-activated protein) kinase, which is found on the tip of the injured axon. Vimentin plays role in transporting the kinase into the body and toward nucleus of cells. The Kinase activates and affects the expression of the neuron gene in such a way that the neurons can respond to the damage. Moon et al (2004) stated that vimentin is a lot of expressed in damaged tissue. Therefore it is suggested that the high expression of vimentin protein in embryo on GD-12 is related with the process of proliferation and migration of cells needed for repairing thelimb buds tissue, as a result of being given with 2-ME. In this study, the giving of 2-ME was done on the 10th days gestation and the observations were made on the 12th. 48 hours after being given with the 2-ME, which is not the end of the recovery effort, it leads to a very high expression of vimentin and it is indispensable to the process of proliferation in repairing the tissues. The results of the Ruyani’s research (2008) states that after giving 2-ME in Swiss Webster mice on the 10th day with a dose of 10 mmol/kg body weight, the protein profile of the forelimb buds changed. The metabolite result of 2-ME, the MAA, directly effects on gene expression which then also affects the existence of certain proteins. Thus the high expression of vimentin protein is required for tissue repairing due to being given with 2-ME and are associated with functions of the cells during the development stage.

The expression of Neurofilamen-low protein in the treatment group, seemed lower compared to that in the control group. The expression of the three sub-units of neurofilamen varied, both among the population of neurons and the axons in the development stage. The expressions of the three subunits are associated with various functions of the cells during the development stage (Viebhan, 1988). As it is already known that, neurofilamen plays role in the morphogenesis process of the neurons (Matus, 1988; Robinson and Anderton, 1988; Riederer, 1990; Riederer, 1992), mainly to maintain the rigidity of the cell.
that it also serves to guide the intracellular transport into the axons and dendrites. Together with other cytoskeleton proteins, neurofilament functions role to establish and maintain the cells’ shape and to facilitate the transportation of particles and organelles within cytoplasm (Liu et al. 2004). Given the nerve supply and muscle into the limb buds began to occur in the embryo on the 13th day of gestation, the expression of neurofilament-low protein also appears lower in the control group. From the data obtained, it is shown that the expression of neurofilament-low protein got decreased after being given with 2-ME on the 10th day of pregnancy, that was apparently not caused by disruption of RNA and DNA synthesis by 2-ME. The low expression neurofilament-low protein was not caused by the compound 2-ME, considering the fact that the innervation of nerves occurred on the 13th day of pregnancy. While neurofilament medium and neurofilament-high protein began to appear expressed, which tended to increase compared to that of the control group.

CONCLUSION

A single dose of 2-methoxyethanol 7.5 mmol/kg body weight given intraperitoneally on gestation day 10, causes an increase in vimentin protein expression. And the decrease expression in neurofilament protein in the forelimb buds of mice embryo is not caused by 2-methoxyethanol.

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