Original Article

MicroRNAs expression in the Ethylene Glycol Monomethyl Ether-induced testicular lesion

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ABSTRACT — Ethylene glycol monomethyl ether (EGME) induces testicular lesion in rats and human. To investigate miRNAs expression in EGME testicular lesion, miRNA array assay and real-time RT-PCR analysis were conducted by using testis in rats treated with 50 and 2,000 mg/kg EGME for 6 and 24 hr. The expression of corresponding target gene for miRNAs was also examined. At 50 mg/kg, there were no changes in the gene expression and histopathological examination. At 2,000 mg/kg, slight decrease of pachytene spermatocytes with cell shrinkage and nucleus pyknosis at 6 hr and remarkable decrease (or cell death) of pachytene spermatocytes with Sertoli cell vacuolation at 24 hr were observed. After 24 hr, miR-449a and miR-92a decreased obviously and, miR-320, miR-134 and miR-188 increased, while only miR-760-5p increased after 6 hr. Above these miRNAs are reported to have an important role for spermatogenesis. The gene expression of Bcl-2, target for miR-449a, increased and therefore it is considered anti-apoptotic reaction has started in this stage. The expression of high mobility group AT-hook 2 (target of miR-92a) which regulates histone structure, was increased. Furthermore, histone deacetylase 4, targets for miR-320, was also affected. Above prohibiting apoptosis or activating epigenetic genes might be protective reaction to spermatocytes death under the miRNAs regulation in EGME testicular lesion.

Key words: EGME, miRNA, Testicular lesion

INTRODUCTION

Ethylene Glycol monomethyl ether (EGME) is widely used in organic solvent in paints, printing inks, varnishes, dyes, and resins an additive in airplane deicing solutions (Johanson, 2000). The adverse effects on peripheral blood, testes, and sperm have been reported in human (Johanson, 2000). In the animal studies, EGME induced testicular atrophy and disruption of spermatogenesis (Chapin *et al.*, 1984; Foster *et al.*, 1983). The major target cell is the pachytene spermatocyte and atrophy, condensation of nuclear chromatin or disruption of nuclei in spermatocytes are appeared (Chapin *et al.*, 1984) and Sertoli cells are also affected (Creasy *et al.*, 1986).

As a one of the toxicological mechanism of EGME testicular lesion, apoptosis induction is known. EGME induced oxidative stress leads to increased expression and

activation of proapoptotic factors BAK and BAX, and it leads activation of caspase 3 and finally, cells are apoptotic death (Bagchi and Waxman, 2008). Furthermore, Methoxyacetic acid (MAA, the active metabolite of EGME) increased androgen binding protein and the cyclic expression of androgen receptor in Sertoli cells (Tirado *et al.*, 2003) and these changes in Sertoli cells could contribute to apoptosis induction by MAA. In the other mechanism, change in HSP70-2 expression, molecular chaperon and one of the spermatogenesis important genes, was reported (Fukushima *et al.*, 2005). Furthermore, MAA shown induction of histone hyperacetylation (Wade *et al.*, 2008) and epigenetic change might affect testicular lesion.

Recently, new molecular mechanism has been found in the spermatogenesis. Namely, recent reports have revealed that non-coding small RNAs play important roles in spermatogenesis. In the testis, there are two

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main types of functional non-coding small RNAs, micro RNAs (miRNAs) and Piwi-interacting RNA (piRNA) (He *et al.*, 2009). MiRNA is transcribed from genome DNA as pre-miRNA and then, mature-miRNA is converted by RNase III Dicer (Lee *et al.*, 2003). MiRNAs play important roles in gene expression such as translation or degradation (Carthew, 2006; Pilla *et al.*, 2007). The number of miRNAs is suggested to be up to 1,000, but each miRNA is assumed to have hundreds of target mRNAs (Lewis *et al.*, 2003). Furthermore, miRNAs are expressed at several organs and are considered to regulate about 30% of all genes in human (Lewis *et al.*, 2005). On the other hand, another type of non-coding RNA, piRNAs are expressed only in germ cells while miRNAs expressed at many organs, and they work for germline development (Klattenhoff and Theurkauf 2008). Currently, 50,000 piRNAs are identified, and it is speculated that total number is approximately 200,000 (Betel *et al.*, 2007). The function of piRNAs is not well known in detail, they are considered to have a role for repression of retrotransposons regulation (Aravin *et al.*, 2007).

Although roles of miRNAs in the spermatogenesis are not well understood so far examined in the literatures, several investigations have been conducted in this respect. Hayashi *et al.* (2008) have reported that miRNAs are important for the proliferation of primordial germ cells and spermatogonia. Marcon *et al.* (2007) identified 37 most abundantly expressed miRNAs in the testis-specific cell types and they illustrated the abundance and variety of miRNAs that can be detected in meiotic prophase in mice. Yan *et al.* (2009) have shown that 26 miRNAs identified are very important for spermatogenesis in developing primates. Furthermore, in knock out mouse of Sertoli cell Dicer, which was induced by disorder miRNA expression in Sertoli cells, infertility have been observed due to complete absence of spermatozoa and progressive testicular degeneration (Papaioannou *et al.*, 2009). The above described new findings mean that miRNAs are essential for all cell types in the testis during spermatogenesis.

The present studies were aimed at investigating miRNAs expression by using miRNA array screening and real-time RT-PCR analysis in the testicular lesion induced by EGME. Furthermore, we also confirmed histopathological changes and the expression of predicted target of miRNA by real-time RT-PCR.

MATERIALS AND METHODS

Animals and administration of EGME

All rat experiments were conducted under the control

of the Committee Regulation of Animal Care and Welfare of Showa University. Twelve-week-old male Crj:CD(SD) IGS rats (n = 30) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The animals were acclimated to our laboratory for 7 days prior to dosing and then randomly assigned to control group and ethylene glycol monomethyl ether (EGME) group according to their body weight. Every one or two animals were housed in one cage and maintained under controlled conditions of light (12 hr light and 12 hr dark cycle) and temperature (19 to 23°C) and allow to eat pelleted feed and drink municipal drinking water *ad libitum*. Ten rats were administered EGME (Wako, Osaka, Japan) once by oral gavage at a dose of 50 and 2,000 mg/kg, respectively. Ten males were administered the vehicle, distilled water, in the same manner and served as control. The dose levels of EGME were set as no observed adverse effect levels and toxicological levels based on our previous studies (Fukushima *et al.*, 2005; Yamamoto *et al.*, 2005). Six and 24 hr after dosing, all animals were euthanized under anesthesia with pentobarbital sodium, and were necropsied and testes were collected from all animals. After the testes were weighed, the right testis was fixed with 10% neutral buffered formalin (Wako) for pathological examination and the left testis was frozen by liquid nitrogen and stored at -80°C until use for gene expression analysis. All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Showa University (#20052).

Pathological examination

The fixed testes were trimmed, dehydrated, embedded in paraffin, sectioned, mounted on glass slides, stained with hematoxylin and eosin and examined by light microscopy.

RNA extraction

From the frozen testes, total RNA including miRNA was extracted using miRNeasy Mini kit (Qiagen, Tokyo, Japan). The quality of purified RNA was checked with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

MiRNA microarray analysis in the testes from rat given EGME at 2,000 mg/kg

Ten mg of frozen testes from each animal per each group was pooled and then, total RNA including miRNA was extracted in the above methods. The microarray analysis was performed using Agilent Rat miRNA array G4473A according to the manufacture's instructions. This array carried total 350 miRNAs. One hundred ng of total

RNA including miRNA was used as template RNA. The RNA was labeled with Cyanine 3-Cytidine bisphosphate (Agilent Technologies) and hybridized to the array. The signal intensity was scanned with Agilent DNA Microarray Scanner G2505B (Agilent Technologies) and then, was calculated using Feature Extraction 9.0 (Agilent Technologies). The microarray analyses were duplicated as shown experiment 1 and 2.

Tissue distribution of affected miRNAs by EGME in the normal animals

To investigate the tissue distribution of affected miRNAs by EGME, liver, kidney, heart, lung, spleen, mesenteric lymphocyte node, and thymus were also collected from different two control animals. For RNA extraction, 50 mg of each frozen tissue was used in each animal. RNA extraction and microarray analysis were individually conducted in the same manners described above. Furthermore, to visualize the tissue distribution, hierarchical clustering analysis using Spotfire® DecisionSite® version 9.1.1 (Spotfire, Tokyo, Japan) was performed using microarray data.

Bioinformatic analysis in the affected miRNAs by EGME

To predict target mRNAs for affected miRNAs by EGME, we used TargetScanHuman version 5.1 (<http://www.targetscan.org/>) and PicTar (<http://pictar.mdc-berlin.de/>). We selected target genes that were commonly picked up in both analyses.

Quantitative real time-RT PCR for miRNA and target mRNA

For miRNA expression, 10 ng of total RNA including miRNA extracted from testis of each animals was reverse-transcribed individually with a TaqMan® MicroRNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA) and TaqMan® MicroRNA Assay containing specific primers of miR-134, miR-188, miR-320, miR-760-5p, miR-92a, miR-449a and U6 snRNA (Applied Biosystems). After that, 1.33 µl of RT sample of each and TaqMan® Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) were applied to real-time RT-PCR. The expression of miRNAs was normalized with each of U6 snRNA expression. For mRNA expression, 1 µg of total RNA was reverse-transcribed individually with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and TaqMan® Gene Expression Assays (Applied Biosystems) including gene-specific probes and primer pairs, *inhibin beta B*; Rn01753772_m1, *caspase 2*; Rn00574684_m1, *B-cell CLL/lymphoma*

2 (Bcl-2); Rn99999125_m1*, *high mobility group AT-hook 2*; Rn00583330_m1*, *histone deacetylase 4*; Rn01427053_m1*, *H3 histone, family 3B*; Rn00821572_g1* and *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (TaqMan® Rodent GAPDH Control Reagents, Applied Biosystems) were used for real-time RT-PCR. The expression of mRNAs was normalized with each of *GAPDH* expression. The Applied Biosystems 7900HT Fast Real-Time PCR system was utilized for real-time RT-PCR. The PCR data were analyzed using 2-ΔΔCT method (Livak and Schmittgen, 2001).

Statistical analysis

MiRNA and mRNA expression data were statistically analyzed by using Dunnet's *t*-test (Dunnet, 1964). A probability of less than 5% was considered to be statistically significantly different.

RESULTS

Histopathological changes in the EGME treatment groups

There were no changes in testicular weight of EGME treatment groups when compared to the control (Data not shown). There were no remarkable pathological changes in the testis of rats treated with EGME at a dose of 50 mg/kg (Data not shown). On the other hand, EGME at a dose of 2,000 mg/kg produced slight depletion of pachytene or diplotene spermatocytes when examined at 6 hr after its dosing. Additionally, EGME caused atrophy, condensation of nuclear chromatin and/or disruption of nuclei in spermatocytes. At 24 hr after the high dose of EGME, similar but severe findings were noted in spermatocytes. Under the same experimental conditions, vacuolation of Sertoli cells was also found. Fig. 1 shows representative histopathological changes at EGME 2,000 mg/kg.

Changes in testicular miRNAs after EGME treatment by using microarray screening

To examine the changes in miRNA expression affected by EGME at 2,000 mg/kg, we firstly conducted microarray analysis using Agilent Rat miRNA array G4473A. We selected miRNAs altered by EGME treatment with the following categories; increased miRNAs: more than 2-fold expression changes from the control in both experiment 1 and 2 at 24 hr, decreased miRNAs: less than 0.6-fold from the control. As shown in Table 1, we found that there were the increase in 7 miRNAs and the decrease in 10 miRNAs at 24 hr after EGME dosing although only one miRNA, miR-760-5p increased after 6 hr of dosing. Especially, miR-134 and miR-188 showed dras-

Fig. 1. Representative photos of rat testes at 2,000 mg/kg EGME stained with hematoxylin and eosin. Arrow heads stand for loss or degeneration of spermatocytes and arrow stands vacuolation of Sertoli cells. Scale bar indicates 50 μ m.

tic increase at 24 hr while their expression in the control group was trace level. Furthermore, many miRNAs belongs to miR-17-92 cluster, miR17-5p, miR-18a, miR-19a, miR-20a, miR-92a, miR-93, were down regulated at 24 hr.

Comparative expression of affected miRNAs by EGME in several normal tissues using miRNA array

Figure 2 showed miR-449a, down regulated by EGME, specifically highly expressed in normal testis while the expression was small or trace level in other tissues examined. On the other hand, the up-regulated miRNAs such as miR-134, miR-188, miR-320 and miR-760-5p showed low expression in all tissues.

Confirmation of miRNA expression by quantitative real-time RT-PCR

In the present study, we measured the expression of 6 miRNAs (miR-134, miR-188, miR-320, miR-760-5p, miR-92a and miR-449a) because the large expression changes were seen after EGME administration (Fig.3). There were no changes at 50 mg/kg of EGME in all miRNAs examined. At 2,000 mg/kg of EGME, the results were well correlated with array analysis. At 6 hr, miR-760-5p showed approximately 4-fold increase when compared to the control. At 24 hr, 2 miRNAs, miR-134 and miR-188, were 10-fold increase of control and miR-320

Fig. 2. Tissue distribution of affected miRNAs by EGME in the control animal. The bar means signal intensity. The expression of miR-449a was specifically high in the testis, while the expression of other miRNAs affected miRNAs by EGME was low in many organs.

also increased while there were no statistically significant. On the other hand, miR-449a and miR-92a halved at 24 hr.

Prediction of target genes based on the web-based analysis

To predict target genes of the above 17 miRNAs, we used TargetScan and PicTar web-data base. PicTar can predict only human annotated miRNAs. Basically, we selected miRNA target genes that were commonly extracted from both web-data. Table 2 shows selected miRNAs and their integrated target genes in relation to spermatogenesis. The target genes belong to 7 main spermatogenesis-related categories including apoptosis, DNA/histone methylation, histone acetylation, histone deacetylation, chromosomal structure, hormone and transcription/signal transduction. miR-499a is predicted to regulate *caspase2* and *B-cell CLL/lymphoma 2 (Bcl-2)* as

Effects of EGME on testicular miRNA expression

Table 1. Signal intensity and fold change of affected miRNAs by EGME 2000 mg/kg

miRNAs	6 hr						24 hr					
	Control			EGME			Control			EGME		
	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2	Exp.1	Exp.2
rno-miR-125a-3p	4.26341	5.4619	6.29959	4.81638	1.5	0.9	6.31149	5.40721	58.3655	57.8091	9.2	10.7
rno-miR-134	-0.31102	-0.29812	4.9083	2.18122	N.A.	N.A.	-1.01779	-0.55894	10.3197	8.94809	NA	NA
rno-miR-188	11.4419	13.0342	18.6734	19.769	1.6	1.5	11.6001	12.0628	146.997	138.822	12.7	11.5
rno-miR-320	4.98037	5.93261	8.03961	7.52034	1.6	1.3	5.12941	4.80687	12.5041	11.8598	2.4	2.5
rno-miR-451	11.8319	12.598	11.8404	12.7682	1.0	1.0	7.69564	7.83344	20.2068	19.1565	2.6	2.4
rno-miR-760-5p	-3.17903	-2.19975	18.6309	18.2798	NA	NA	-3.04803	-4.78118	2.09532	2.20528	NA	NA
rno-miR-874	-3.60716	-4.32611	-1.37374	-2.18496	NA	NA	-4.24948	-4.14635	11.2208	10.3078	NA	NA
rno-miR-17-5p	13.4619	19.3852	11.1014	14.7362	0.8	0.8	18.6515	16.7536	6.78099	5.31009	0.4	0.3
rno-miR-18a	67.9449	72.3895	63.995	74.3384	0.9	1.0	81.2593	80.8107	38.9178	40.2864	0.5	0.5
rno-miR-19a	76.8407	94.7181	67.1407	82.0673	0.9	0.9	92.9582	89.676	54.349	51.4347	0.6	0.6
rno-miR-20a	506.172	510.438	504.403	562.322	1.0	1.1	619.63	616.238	307.938	300.805	0.5	0.5
rno-miR-92a	431.203	415.618	451.396	478.061	1.0	1.2	503.868	492.845	228.574	223.364	0.5	0.5
rno-miR-93	28.0779	36.4968	24.8624	30.9271	0.9	0.8	32.2802	31.1507	16.7183	15.3826	0.5	0.5
rno-miR-106b	149.053	147.066	146.904	155.885	1.0	1.1	159.874	155.017	92.2896	89.1372	0.6	0.6
rno-miR-141	4.23435	3.49439	2.12967	2.46585	0.5	0.7	4.4039	2.75438	1.73776	0.607112	0.4	0.2
rno-miR-363	8.10189	8.42039	7.63756	6.45406	0.9	0.8	9.4057	6.86984	4.50085	2.53667	0.5	0.4
rno-miR-449a	621.646	715.72	525.545	603.759	0.8	0.8	680.874	648.983	381.439	356.692	0.6	0.5

Note. Total RNA from 5 animals per each group were pooled and applied to microarray analysis. The microarray analysis was duplicated, experiment 1 and 2. NA means “not applicable” because of “-” minus signal.

Table 2. Putative target genes for affected miRNAs by EGME

miRNAs	Target genes				
	Apoptosis	Epigenetic regulation	Hormone	Transcription factors and others	Others
miR-125a-3p	-				
miR-134	-	HDAC5	-	-	-
miR-188	-	H3F3B	-	-	-
miR-320	-	HDAC4	-	-	-
miR-451	-				
miR-760-5p	-	HDAC7,JMDJ3, MYST2	-	-	NASP
miR-874	-				
miR-17-5p, miR-20a miR-93, miR-106b	-	MECP2,HDAC4, HMGA2	-	FOXJ2	RSBN1
miR-18a	-	EMHT1	-	-	-
miR-19a	MCBE2	HDAC4	INHBB	SOX6	TESK2
miR-92a, miR-363	-	HMGA2	-	KLF4, NOTCH1, MTIF	RSBN1, TSGA 14, SSFA2
miR-141	-	HDAC4	-	-	SSFA9
miR-449a	CASP2, BCL2	-	INHBB	FOXJ2, NOTCH1, KITLG	-

Note. Putative target spermatogenesis-related genes were selected from the data of TargetSan 5.1analysis and PicTar. Regarding miR-760-5p, PicTar could not be used because this miRNA was not annotated in human. Abbreviations were follows. BCL2:B-cell CLL/lymphoma 2. CASP2:caspase 2. EHMT1: euchromatic histone-lysine N-methyltransferase 1. FOXJ2: forkhead box J2. H3F3B: H3 histone, family 3B. HDAC5: histone deacetylase 5. HDAC4: histone deacetylase 4. HMGA2: high mobility group AT-hook 2. INHBB: inhibin, beta B. JMDJ3: jumonji domain containing 3, histone lysine demethylase. KITLG: KIT ligand. KLF4: Kruppel-like factor 4. MECP2: methyl CpG binding protein 2. MITF: microphthalmia- associated transcription factor. MIST2: MYST histone acetyltransferase 2. NOTCH1: Notch homolog 1. NASP:nuclear autoantigenic sperm protein. RSBN1: round spermatid basic protein 1. SOX6: SRY (sex determining region Y)-box 6. SSFA2:sperm specific antigen 2. SSFA9: sperm specific antigen. TESK2: testis-specific kinase 2. TSGA14: testis specific, 14.”-“ means no target.

Effects of EGME on testicular miRNA expression

Fig. 3. Changes in testicular miRNAs expression by EGME. The expression of the 6 miRNAs was confirmed using real-time RT-PCR. The asterisk (*) indicates significant difference from the control group in each time point ($P < 0.05$). The asterisk (**) indicates ($P < 0.01$).

apoptosis-related genes and furthermore, this miRNA has a target for hormone-related gene, *inhibin beta B*. Interestingly, many miRNAs changed by the administration of EGME, such as miR-760-5p, miR-17-5p, miR-20a, miR-93 and miR-106b, miR-92a, miR-19a and miR-141, targeted DNA/histone methylation-related gene, *methyl CpG binding protein 2*. Likewise, several miRNAs such as miR-134, miR-141, miR-188, miR-320, miR-363 and miRNAs including miR-17-92 cluster had target genes associated with chromosomal structure- or histone acetylation/deacetylation-related genes such as *H3 histone, family 3B*, *high mobility group AT-hook 2* or *histone deacetylase*. Moreover, miR-449a and miR-17-92 cluster also had targets related to meiosis such as *forkhead box J2*, *SRY (sex determining region Y)-box 6*, *Kruppel-like factor 4*, *microphthalmia-associated transcription factor* and *Notch homolog 1*.

Confirmation of the gene expression of predicted targets for affected miRNAs by quantitative real-time RT-PCR

In the present study, as predicted targets for miR-449a, the apoptosis-related genes, *caspase 2* and *bcl-2*, and hormone-related gene, *inhibin B* were measured. Additionally, epigenetic-related genes such as *high mobility group AT-hook 2* (target of miR-92a) or *histone deacetylase 4* (target of miR-320) were also measured. Figure 4 showed the expression of target genes. At 50 mg/kg, there were no changes in the gene expression examined except for *caspase 2*. At 2,000 mg/kg, *caspase 2* increased after 6 hr of dosing and after 24 hr, the expression went back normal level. The expression of *bcl-2* was tended to increase after 6 and 24 hr after dosing. Regarding *high mobility group AT-hook 2*, the expression was increased after 6 and 24 hr. On the other hand, the expression of *histone deacetylase 4* decreased after 24 hr and *H3 histone, family 3B* tended to decrease after 24 hr.

Fig. 4. Changes in testicular mRNAs expression by EGME. The expression of the 6 mRNAs was confirmed using real-time RT-PCR. The asterisk (*) indicates significant difference from the control group ($P < 0.05$). The asterisk (**) indicates ($P < 0.01$).

DISCUSSION

At EGME 2,000 mg/kg, typical lesion was observed while there were no remarkable findings at 50 mg/kg. After 6 hr of dosing, slight condensation of nuclear chromatin and/or disruption of nuclei in spermatocytes were observed. After 24 hr, spermatocyte death was observed and, additionally, vacuolation of Sertoli cells were also noted. EGME is well known to induce apoptosis of spermatocytes and cell death (Suter *et al.*, 1998). In fact, the expression of *caspase 2*, activator of apoptosis, increased at 6 hr when spermatocytes death was found histologically. Therefore, this increase is considered to result in spermatocytes with disruption of nuclei and/or condensation of nuclear chromatin. Furthermore, we suggest Sertoli vacuolation also affected spermatocyte death as a secondary effect.

In the present study, to investigate affected miRNAs in the EGME induced testicular toxicity, miRNA array analysis was conducted using testes collected from rats giv-

en at EGME 2,000 mg/kg as a 1st screening. As a result, at 24 hr, the expression of 7 miRNAs increased and 10 miRNAs decreased while only one miRNA affected at 6 hr. The array screening revealed drastic changes in miRNA expression at 24 hr. To confirm the array results, real-time RT-PCR analysis was conducted for several affected miRNAs. The data of PCR analysis was well correlated with miRNA array analysis. At 2,000 mg/kg, dosage that severe morphological changes were noted, drastic increases of expression up to 10 folds of control were noted in miR-760-5p, miR-188, miR-134, miR-320, and decreases was noted in miR-449a and miR-92a. However, at 50 mg/kg, the dosage that there were no remarkable findings in histopathology, any miRNAs were not affected. Therefore, these results indicate the expression of miRNAs was affected by EGME and it is considered to reflect on the lesion. Several papers reported some of the above miRNAs had important roles for spermatogenesis. Marcon *et al.* identified 37 most abundantly expressed miRNAs including miR-320, miR-134 and miR-188 in

the testis-specific cell types such as spermatocyte, spermatogonia and they illustrated the abundance and variety of miRNAs that can be detected in meiotic prophase in mice (Marcon *et al.*, 2007). Current results showed the expression of above 3 miRNAs increased about 10-fold and the changes were remarkable, therefore, these miRNAs is considered to have an important role for testicular lesion induced by EGME. On the other hand, Yan *et al.* (2009) reported the expression of miR-449a were high in mature monkey and human. This data supports miR-449a is one of the important miRNAs for spermatogenesis. The present results of tissue distribution for affected miRNAs by EGME produced miR-449a was specifically high expressed in the testis in normal animals when compared to the other tissues. This data can also support miR-449a is considered to have an important role in the testis. Another decreased miRNA by EGME, miR-92a, belongs to miR-17-92 cluster. In this study, many other miRNAs such as miR-17-5p, miR-18a, miR-19a, miR-20a, miR-93 and miR-106b belong to miR-17-92 cluster and they were down-regulated by EGME. Hayashi *et al.* (2008) showed miR-17-92 cluster are important role for the proliferation of primordial germ cell and spermatogonia. From the evidences, down regulation of miR-17-92 cluster might affect spermatogonia proliferation.

Currently, each miRNA is assumed to have hundreds of target genes (Lewis *et al.*, 2003). Therefore, even though only one miRNA expression is changed, it gives big impact on the regulation of target genes. From mentioned above, EGME affected several miRNAs and thus, we considered there was big impact on target mRNA expression. Unfortunately, present study could not show direct regulation of miRNA and mRNA, but we could report indirect regulation using in silico target predicted system by Target Scan or Pic Tar.

The gene expression of *inhibin B*, one target for miR-449a, was not affected by EGME. However, methoxyacetic acid, a major metabolite of EGME, induced significant increases in inhibin level of blood and testicular interstitial fluid (Maddocks *et al.*, 1992). Therefore, miR-449a suggests regulating inhibin in translational level. Such alteration of inhibin expression might be associated with Sertoli cell vacuolation and cell death induced by EGME.

The other target for miR-449a, *bcl-2* tended to be increased by EGME after 24 hr of dosing. Bcl-2 is known to have a role for inhibition of apoptosis. Due to the suppression of miR-449a expression by EGME, it was speculated that *bcl-2* regulation by miR-449a became weak and then, the transcription of *bcl-2* was increased. The regulation of *bcl-2* by miR-449a might be considered prohibiting apoptosis induced by EGME.

The affected miRNAs such as miR-320, miR-134, miR-188, miR-760-5p and miR-92a have targets for many epigenetics-regulated genes. The predicted target of miR-320 is histone deacetylase 4 (HDAC4) and the gene expression decreased at 24 hr after EGME 2,000 mg/kg of dosing. MAA induced histone hyperacetylation and the mechanism was considered to activate histone acetylase and inhibit enzyme activity of HDAC (Wade *et al.*, 2008). The present result revealed HDAC4 were also suppressed by EGME in gene expression level and this finding was well correlated with previous report. Therefore, the down regulation of HDAC4 is speculated to be controlled by the up-regulation of miR-320. Currently, it is unclear the relationship between histone hyperacetylation and spermatocyte death but, histone acetylation results in changes in chromosomal structure and up-regulation of gene transcription. Thus, we speculated this histone modification led to activate proliferation or meiosis rather than leading to spermatocyte death. In current study, the expression of other epigenetic-related gene, *high mobility group AT-hook 2 (HMGA2)*, was measured. HMGA2 predicted target for miR-92a and the gene expression increased at 24 hr after EGME 2,000 mg/kg dosing. HMGA2 play a role in determination of chromatin structure and regulating the transcription and the protein is expressed during the process from spermatocyte to spermatid (Chieffi *et al.*, 2002). In the knock out mice of HMGA2, meiosis was disordered and finally, spermatozoa was absent in the testis (Chieffi *et al.*, 2002). Administration of EGME at 2,000 mg/kg produced severe depletion of spermatocytes in the present study. The increase in *HMGA2* is considered to repair reaction to spermatocyte death as EGME toxicity and the increased expression might contribute to activate meiosis. The up-regulation of HMGA2 by down regulating miR-92a might be repair reaction to EGME toxicity. In the present study, there were many other epigenetics-related target genes such as H3 histone, family 3B (predicted target of miR-188), MYST histone acetyltransferase 2 (miR-760-5p) or methyl CpG binding protein 2 (miR-17-5p etc.) and these putative targets have been reported to be important for spermatogenesis (Jiang *et al.*, 2002; Tachibana *et al.*, 2007). The gene expression of *H3 histone, family 3B* was not markedly changed in the PCR analysis but, it tended to decrease. The up-regulating miR-188 by EGME may affect decrease in the expression of *H3 histone, family 3B*. DNA or histone modification is very important for spermatogenesis (Trasler, 2009) and miRNAs may have an important role for epigenetic regulation.

In conclusion, many miRNAs were affected in EGME induced testicular lesion. Especially, miR-188, miR-320

and miR-134 drastically increased and miR-92a and miR-449a decreased. These miRNAs have shown to have roles for spermatogenesis. Based on the gene expression analysis of putative targets of affected miRNAs, we speculated anti-apoptotic reaction and activation of epigenetic related genes were happened after 24 hr of dosing. These changes in miRNAs and mRNAs might be protective or repair reaction against spermatocyte death by EGME. The current study did not explain direct regulation of miRNAs and target genes and therefore, further investigation is needed. However, this study can provide clues of roles for miRNAs in EGME induced testicular toxicity.

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