UTILIZATION OF MAGNETIC RESONANCE MICROSCOPY IN REPRODUCTIVE TOXICOLOGY

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Introduction

Several compounds, including endocrine disruptors, have been shown to impair reproductive functions in mammals. In some cases, they have acute effects on gametogenesis in mature animals and viability of embryos. However their actions are often chronic, especially at low doses, and may even affect animals in the early stages of development causing malfunctions after maturation. In these cases, continuous observation of the pathogenic process *in vivo* will be helpful to clarify the mechanism of the toxicity. Several techniques for *in vivo* imaging have been developed. Magnetic resonance imaging (MRI) is a useful technique for non-invasive investigation and enables longitudinal examination of internal organs in humans and animals. In addition, MRI allows mapping of chemical composition, known as spectroscopic imaging or chemical shift imaging, and localization of specific objects by labeling techniques. Magnetic resonance (MR) microscopy is an MRI method with enhanced spatial resolution. In MR microscopy, spatial resolution is enhanced to sub-millimeter orders by the use of a strong static magnetic field and high magnetic field gradients [1,2], while that in traditional MRI, which is widely used in the clinical field, is of the order of 1 mm. Using this technique, non-invasive and multi-dimensional observation of small specimens can be performed at a level necessary for investigation of embryo morphology in small animals. In this study, we applied MR microscopy to assess the morphology of murine embryo specimens and *in vivo* imaging in immature postnatal mice. Detection of changes induced by exposure to chemicals was also attempted.

Materials and Methods

Imaging of embryo specimens

Embryos at 15.5 to 18.5 dpc were collected from pregnant ICR mice. As a model for transplacental teratogenesis, induction of embryo malformations by administration of retinoic acid was performed as described previously [3]. Briefly, all-trans retinoic acid (RA) dissolved in dimethyl sulfoxide (DMSO) was intraperitoneally injected into pregnant mice (50 mg/kg body weight) at 8.5 dpc. Control mice were given DMSO without RA. Diethylstilbestrol (DES) and bisphenol-A (BPA) were used as compounds relevant to reproductive toxicology. DES dissolved in corn oil was injected subcutaneously into pregnant mice (100 μg/kg body weight/day, from 8.5 dpc to the day before sampling). BPA was given by subcutaneous administration (20 μg or 200 mg/kg body weight/day, from 8.5 dpc to the day before sampling) or oral gavage (2 μg/kg body weight/day, from 6.5 dpc to the day before sampling). Control mice received vehicle solution by the same procedure. Collected embryos were fixed in 80 % ethanol or 10-20 % formalin. Fixed embryos were examined using an MR microscope, with a 400 MHz wide bore spectrometer equipped with a microimaging attachment (NM-AIM imaging equipment, JEOL, Japan). Each embryo was embedded in 3% low melting point agarose contained in a sample tube. Embryos were set into an imaging probe at the center of the detection coil (20 mm in diameter). The imaging probe was inserted into a magnet with a static magnetic field of 9.4 T equipped with coils producing magnetic field gradients (11.75 G/cm for each axis). Proton MR microimages were acquired using two-dimensional or three-dimensional Fourier transformation spin echo (2DFT-SE and 3DFT-SE, respectively) sequences. Sizes of the field of view (FOV) were 30 mm for each axis in 18.5 dpc embryos and 20 mm in other stages. In 2DFT-SE, five slices 1 mm thick were acquired with 1 mm center-intervals using a multislice spin-echo sequence. Different values of repetition time (TR) and echo time (TE) were used to examine their effects on images.

In vivo imaging of immature mice

Immature mice (1 to 3 weeks old) derived from normal or BPA-treated dams (oral gavage, 2 μg/kg body weight/day, from 6.5 dpc to term) were used. Mice were anesthetized by intraperitoneal injection of Nembutal and placed in the detection coil in the imaging probe of the MR microscope. To observe the reproductive tract, lower abdominal region was located at the center of FOV. Proton MR microimages were acquired using 2DFT-SE and 3DFT-SE sequences as described for observation of embryo specimens. The size of the FOV was 20 mm for each axis.

Results and Discussion

Examination of RA-treated embryos

Administration of RA during pregnancy has been suggested to induce widespread malformation in developing mammalian embryos. We previously reported that a single-shot maternal administration of RA caused malformations in murine embryos showing characteristic defects in the skeleton depending on dose and developmental stage of embryos at the time of treatment [3]. In embryos given RA at 8.5 dpc, malformations were observed in the craniofacial and trunk region. Disappearance or severe hypoplasia of the tail occurred in a high proportion of embryos. In the skeletal preparations of these embryos, serious malformations were observed in vertebrae posterior to the lumbar vertebra. Administration of DMSO caused no notable malformations.

The morphological properties of RA-treated embryos could be clearly visualized by MR microscopy. On 2DFT-SE images with 256 x 256 matrices, corresponding to an in-plane resolution of 117 μm for FOV of 30 x 30 mm and 78 μm for FOV of 20 x 20 mm, major organs appearing in sagittal sections of embryos could be recognized. TR and TE influenced the contrast of images. Acquisitions of images with a short TR enhanced the skeletal system. On images with TR/TE = 500/5 (msec), almost the whole skeleton showed stronger signals compared with other tissues. On images with TR/TE = 200/5, separate regions in the skeleton showed high signal intensities. Representative areas showing strong signals in the skeleton were located in the snout, basis cranii and spine. In vertebrae, stronger signals appeared as regular bands across the spine. On Images with long TE (TR/TE = 1000/10 or 1000/20), connective tissue including the skeletal system showed relatively weak signals. The heart and liver also showed weak signals. This may have been due to T2 shortening effects of paramagnetic iron derived from hemoglobin. Skeletal defects were detected in 2DFT-SE images of 18.5 dpc RA-treated embryos. Protrusion of the spinal cord was observed in some cases with defects in caudal vertebrae. Similar findings were also observed in 15.5 dpc embryos given RA and confirmed histologically. Changes in the morphology of the digestive tract and a- or hypogenesis of kidneys were also noted in RA-treated embryos, although the typical pattern has not yet been defined. On 3DFT-SE imaging, T1-weighted images were observed with TR/TE = 200/5 msec, which was expected to emphasize the skeletal system based on the results of 2DFT-SE imaging. Morphological changes by RA treatment were also demonstrated in three-dimensional images reconstructed from 3DFT-SE data with 128^3 matrices. Although projection images can also be made from multislice 2DFT-SE images, gaps often occur between neighboring slices to avoid cross-talk artifacts. Structural details may fail to be detected when they are located in the gaps. Although three-dimensional data require longer times for acquisition and higher capacity for processing, such techniques will be useful for the assessment of multidimensional structures.

Examination of embryos exposed to DES or BPA.

It has been reported that *in utero* exposure to DES causes defects in development of reproductive organs[4-11], for example, failure of testis descent in male embryos. On transverse images of the abdominal region of male 18.5-dpc embryos acquired by multislice 2DFT-SE, testes could not be visualized. 3DFT-SE images with 128^3 matrices were not sufficient and 256^3 matrices were needed for detection of the testes. To acquire sufficient signal intensity from the abdominal organs, TR had to be increased to 300 msec rather than 200 msec used in skeletal imaging. MR microimages showed that the testes remained at a higher position in the abdominal cavity in some embryos exposed to DES, while they were located at the same level as the bladder in control embryos. Delay or defects in testis descent were also observed in 15.5-dpc embryos obtained from mice treated with at 200 mg/kg body weight/day of BPA by subcutaneous injection. At this stage of development, however, considerable individual differences of testes location were observed even in normal embryos, and therefore further studies are necessary to determine the effects of BPA. No notable differences were observed in embryos given lower doses of BPA by oral gavage compared with controls. The reproductive tract in female embryos could not be visualized by the imaging techniques used in this study.

These results suggested that a spatial resolution of 100 μm or better is needed for assessment of reproductive organs in mouse embryos. To acquire this level of resolution in living embryos *in utero* within a shorter time frame, data acquisition should be performed using fast imaging techniques such as the fast low angle shot (FLASH) technique [12] or echo planar imaging [13].

In vivo imaging of immature mice

Tissue in living mice showed weaker signals than fixed specimens with the same TR and TE. T1 of testes *in vivo* was estimated to 1500 to 2000 msec from images. Images with 256^2 matrices by 2DFT-SE had too much noise to allow examination of soft tissue even at TR = 4000 msec. Reduction of data matrices to 128^2 improved image quality, although details were blurred. Most of the tissues showed similar signal intensity except low signal areas corresponding to bones in proton density-weighted images (TR/TE=4000/5). In T2-weighted images (TR/TE=4000/20) of male mice, testes showed high signal intensity compared to skin, muscle and other connective tissue. Adipose tissue demonstrated high signal intensity in T2-weighted and in T1-weighted images (TR/TE=1000/5). In 3DFT-SE imaging, T1-weighted sequence (TR/TE=300/5) was used to minimize the observation time. Images acquired by this procedure, however, did not have sufficient tissue contrast. Female reproductive organs could not be visualized clearly. This was probably due to their relatively small size and relaxation properties similar to surrounding tissue in the animals examined in this study.

Serial observation of testes in immature mice was performed using multislice 2DFT-SE

sequences. Testes were detected in the abdominal cavity at postnatal day 15 or earlier. Descent of the testes into the scrotum was observed in mice at day 18 or later. Signal intensity was slightly higher in the descended testes compared to those in the abdominal cavity; however, this may have been due to a partial volume effect. In some mice, paired areas of low signal intensity were observed in the caudal and medial parts of the scrotum or processus vaginalis of the peritoneum. These were considered to correspond to the cauda epididymidis and ductus deferens. There were no significant differences between untreated mice and those exposed to BPA *in utero*. Slice intervals in the present technique may have been too large to examine organs in neonates. It is necessary to improve three-dimensional imaging techniques for precise determination of the size and position of the organs.

The Results of the present study showed that developing organs in mice can be examined *in vivo* by MR microscopy. This technique will serve as a new tool for reproductive toxicological studies providing longitudinal information during ontogeny.

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