Perinatal development of the rat hip joint with restrained fetal movement

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ABSTRACT We compared the structures of the femoral head (FH) of neonates between normal and operated legs with restrained fetal movement using an exo utero technique. At embryonic day (E) 16.5, one hind limb was sutured onto the embryonic membrane and the fetuses were allowed to develop exo utero until the term (E22.5). There was no significant difference in the largest diameter of the FH between the non-operated and operated side FH in the operated neonates and the FH of the non-operated neonates. By scanning electron microscopy, roughness and collagen fiber bundles, which were detected on the surface of the operated side FH at E18.5, disappeared at E22.5. However, the operated side FH was deformed and the surface cell arrangement was more irregular than that of the controls at E22.5 by light microscopy. These results suggest that the abnormality of cell arrangement caused by the restraint of fetal movement may induce the deformity and irregularity of the FH surface, although this operation may not disturb the basic cellular activities such as cell proliferation as well as the secretion of cartilage matrix and collagen fibers. To further investigate the recovery process in the operated newborns after releasing the restraint, we bred them artificially for a considerable period after birth. The operated side FH surface of the neonate bred for 45 hours was smoother than that at E22.5 and similar to that of the non-operated side FH. This result suggests that the proper movement of the extremities after birth may recover the deformity caused by restrained fetal joint movement.

Key words: rat neonate, hip joint, fetal movement, exo utero development, congenital dislocation of the hip, light microscopy

INTRODUCTION

Congenital dislocation of the hip (CDH) is one of the most common congenital skeletal deformities. The prevalent type, which constitutes up to 98% of the cases of CDH, is represented at birth by a dysplasia of the hip consisting of a flat acetabular roof and an underdeveloped proximal end of the femur, i.e., rather slight anomalies that predispose to dislocation (Warkany, 1971). The mechanisms of the delayed prenatal development of the hip joint in the patients of CDH still remain unclear. Generating an animal model of CDH is helpful to clarify the etiology and in the development of prevention and treatment. Immobilization of the hip joint in postnatal rats for the dislocation of the hip joint induced progressive acetabular dysplasia and anatomical abnormalities of the head and neck of the femur during the postnatal period (Sijbrandji, 1965). We previously studied the effects of fetal movement of the hind limb on the formation of the hip joint in rats, and reported that the fetal hind limb movement influenced the development of the femoral head (FH) and acetabulum (Kihara et al., 1998). We tied the hind limb on one side onto the embryonic membrane to restrict the range of motion at the hip joint at embryonic day (E) 16.5 using a technique of exo utero development (Muneoka et al., 1986; Hatta et al., 1994a, 1994b; Naruse et al., 1996; Sekimoto et al., 1997; Zhang et al., 1998, Hatta et al., 2002, Matsumoto et al., 2002). The surface of the FH of the operated fetuses was irregular and rugged with banks, and subsurface collagen fibers did not form bundles but remained as a dense meshwork at E 18.5 in light and scanning electron microscopy (LM and SEM) (Kihara et al., 1998). We observed the hip joint of rat fetuses at E18.5 in the previous study; however, the findings were not sufficient to evaluate the effect of fetal movement on the development of the hip joint till term or to analyze the results correlatively with those from postnatal studies.

In this study, therefore, we observed the hip joint of operated neonates at E22.5, with restricted movements of the hind limb from E16.5, after they were delivered by Caesarian sec-
tion and studied the effect of fetal movement on the development of the hip joint during the fetal period till term. We detected abnormalities of the surface structure of the femoral head caused by the restriction of the fetal limb movement. Additional study with artificially fed offspring also revealed the recovery of those abnormalities induced by postnatal leg movement.

MATERIALS AND METHODS

Morphological analysis of the normal development of the hip joint in rats

The animals used in this study were Jcl:Wistar white rats (CLEA Japan Inc., Tokyo, 9-15 weeks of age), which were maintained at the Institute of Experimental Animals of Shimane Medical University and handled in the following experiments in accordance with the institutional guidelines. An estrous female was placed in the same cage with a potent male in the evening. When a vaginal plug was found the next morning, noon of that day was designated E0.5. The females were sacrificed by an overdose of ether anesthesia and fetuses were obtained at E22.5. Subsequently, the body weight (BW) of the fetuses was measured and the hind limbs of the one side were fixed for observation by LM with 10% formalin neutral buffer solution. The hind limbs of the other side were fixed for electron microscopic observation with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The formalin-fixed specimens were embedded in paraffin and serial sagittal sections (5 µm) were made. The sections were stained with hematoxylin and eosin and observed under a LM. The largest diameter of the FH (LDFH) was measured on the largest section of the FH from the serial sections for each specimen using the NIH Image Software (public domain software, Wayne Rasband, US National Institutes of Health).

The FHs fixed for electron microscopy were prepared for conventional SEM observation (Hirotani and Ito, 1975; Otani et al., 1993). The specimens were postfixed with buffered 1% osmium tetroxide (OsO4) at 4°C and stained with 1% tannic acid solution followed by buffered 1% OsO4. They were dehydrated in a graded series of ethanol, dried with a Hitachi HCP-2, coated with Pt-Pd using an Eiko VX-10A and observed with a Hitachi S-800 SEM at 15 kV.

Fetal operation and exo utero development

The animals used were the same as above. We performed the exo utero operation at E16.5 as in the previous study (Kihara et al., 1998). At E16.5, pregnant rats were anesthetized with 50 mg/kg body weight pentobarbital for the fetal operation. The uterine wall was cut longitudinally at the side opposite the entrance of the uterine artery and the site of the placenta to expose the fetuses covered by the embryonic membrane. The hind limb of one side was bound at the knee joint or more distally to the embryonic membrane with 9-0 thread used for ophthalic surgery, whereas the other side was left unoperated. The hind limbs were tied in situ and were not forced into any specific abnormal positions. The holes made on the embryonic membrane by the suture were closed with hemostatic ox collagen fleece (Alcon, Humacau, Puerto Rico) in order to prevent leakage of the amniotic fluid. We operated on two fetuses located in each side of the uterus horn and the other embryos were removed from the uterus. Fig. 1 shows the tying at the hind limb of an E16.5 rat fetus onto the fetal membrane using a styptic support. Approximately 15 ml of Hank's solution (37°C) was injected into the peritoneal cavity before the closure of the abdominal wall to prevent

Fig. 1 A photograph of an E16 fetus being operated on for restriction of the fetal movement of the hip joint (a). A schema corresponding to the left picture (b). The arrows indicate the thread used for the surgery. The asterisk indicates hemostyptic. The arrowheads indicate the hand. The open arrowheads indicate the blood vessel on the yolk sac. The rectangle over the schema represents the area of the left picture.
adhesion (Hatta et al., 1994b). At E22.5, the dams were sacrificed with an overdose of diethyl ether and the fetuses were obtained. We obtained 12 operated live neonates in total from 10 dams. The operated hip joints of E22.5 neonates were observed with LM and SEM as described above and compared with the non-operated side of the hip joints of the operated neonates and those of neonates developed normally in utero.

Postnatal breeding

In order to examine the effects of leg movement after birth on the abnormalities of the FH induced by the restrained fetal movement, we prepared foster mothers of rats to breed the neonates developed exo utero. However, the foster mothers killed the operated neonates, probably because of the lower activity and smaller size than normal neonates. Therefore, we gave a formula for humans to the operated neonates every 2 hours. They were able to drink it, but could not pass urine. We stimulated the lower part of the abdomen with a wet brush to encourage urination every one hour, and if they didn't move after the stimulate they were immediately sacrificed. We dissected them and found their bladders to be extended. It was suggested that they were suffering from urinary disturbance. We obtained 4 operated neonates which survived for a considerable period of up to 45 hours by this artificial breeding.

Statistical analysis

The numbers of samples examined are shown in Tables 1 and 2. The measured values in Table 1 are described as the mean ± standard deviation and were statistically analyzed by Student's t-test. A level of p < 0.05 was regarded as significant.

RESULTS

The effects of restraint of fetal movement on the hip joint at E22.5

The results of the measurement of BW and LDFH are summarized in Table 1. In the experiment of the fetal operation, a total of 12 live neonates which were operated on at E16.5 were obtained at E22.5. BW of the operated newborns (4.51 ± 0.65 g) was significantly smaller than that of the non-operated neonates (5.35 ± 0.48 g, p < 0.05). However, there was no significant difference between the LDFH of the non-operated (0.86 ± 0.07 mm) and operated side FH (0.84 ± 0.05 mm) in the operated neonates and the LDFH of the non-operated neonates (0.87 ± 0.07 mm).

In the LM study, the shape of the FH in normal neonates at E22.5 was round and the surface was smooth (Fig. 2a). In the surface region of the FH, one or two layers of flat squamous cells existed and two or three layers of spindle or oval shaped cells were underneath the squamous cell layers (Fig. 2b). In the operated side FH of the operated neonates at E22.5 (Figs. 2c and 2d), the shape of the FH was deformed and the FH surface and acetabulum were rough. The arrangement of cells in the surface region was irregular and some of these cells were pyknotic. In the underlying mesenchymal cells, pyknotic

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of neonates</th>
<th>BW(g) (mean ± SD)</th>
<th>LDFH(mm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-operated</td>
<td>14</td>
<td>5.35 ± 0.45</td>
<td>0.87 ± 0.07</td>
</tr>
<tr>
<td>Operated</td>
<td>12*</td>
<td>4.51 ± 0.65</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.86 ± 0.07</td>
</tr>
<tr>
<td># Operated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>side (n = 8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-operated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>side (n = 4)</td>
<td></td>
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</tbody>
</table>

#: This number of samples includes the 4 neonates bred artificially after birth (see Table 2).

SD: Standard deviation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Period of survival (hour)</th>
<th>LDFH(mm)</th>
<th>Methods of the observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>12</td>
<td>0.86</td>
<td>LM</td>
</tr>
<tr>
<td>II</td>
<td>12</td>
<td>0.81</td>
<td>SEM</td>
</tr>
<tr>
<td>III</td>
<td>20</td>
<td>0.99</td>
<td>LM</td>
</tr>
<tr>
<td>IV</td>
<td>45</td>
<td>0.94</td>
<td>LM</td>
</tr>
</tbody>
</table>
cells were more frequently observed in the operated side FH than in the non-operated normal control (Fig. 2d vs. 2b). In the non-operated side FH of the operated neonates (Figs. 2e and 2f), the shape of the FH was not deformed and the surface appeared almost the same as that of the normal neonates (Fig. 2e). One or two layers of squamous cells existed underneath the surface and the irregularity of the cell arrangement like in the operated side FH were not observed (Fig. 2f vs. 2d). When comparing FH of the operated fetuses at the E22.5 with that at E18.5 (Kihara et al., 1998), the shape of the E22.5 FH was slightly deformed (Fig. 2c vs. 2g), and the surface layer at E22.5 was irregular being composed of cells with smaller sized nuclei than that of E18.5 (Fig. 2d vs. 2h). The space between the cells in the E22.5 FH became more extended than that in the E18.5 FH and the cell arrangement underneath the surface of the E22.5 FH was irregular and not a layered structure (Fig. 2d vs. 2h).

In SEM observation, the FH surface of the normal neonates at E22.5 was generally smooth and flat mounds which appeared to correspond to the nuclei of surface mesenchymal cells were scattered on the surface (Fig. 3a). The surface of FH of the operated side at E22.5 was rough and many mounds which corresponded to the nuclei were seen on the surface (Fig. 3b). However, when compared with the operated side FH at E18.5 (Kihara et al., 1998), the surface of FH of the operated side at E22.5 appeared smoother, and the collagen fibers which were observed at E18.5 in the previous study (Fig. 4e in the paper; Kihara et al., 1998) were undetectable on that of the operated neonates at E22.5.

The recovery from the abnormality caused by the restraint of fetal movement after birth

In order to examine the effects of the postnatal joint movement on the abnormality of the operated hip joint, we bred operated neonates artificially. We obtained 4 neonates (I-IV) which survived for a considerable period. The results of the measurement of LDFH of the operated side of these neonates are shown in Table 2. LDFHs of I and III (12 and 20 hours survival, respectively) were within the mean ± standard deviation (SD) of the operated side at birth (Table 1), but the operated side LDFH (0.99 mm) of IV (45 hours survival) was larger than the mean+2SD of the operated side FH in

Fig. 2 Light micrographs of the hip joint of the normal and operated neonates. The hip joint of a normal neonate at E22.5 (a, b). The shape of the FH is round and the surface is smooth (a). In the surface region of the FH, one or two layers of flat squamous cells (open arrowheads) exist and two or three layers of spindle or oval shaped cells (small arrowheads) are underneath the squamous cell layers (b). The operated side hip joint of an operated neonate at E22.5 (c, d). Large arrowheads indicate deformed parts of the FH (e). The arrangement of cells in the surface region is irregular and a part of these cells which are indicated by arrows are pyknotic (d). The non-operated side hip joint of an operated neonate at E22.5 (e, f). The shape of the FH is not deformed and the surface appeared almost the same as that of the normal neonates (a vs. e). The cell arrangement in the surface region of FH is not irregular like in the operated side FH (f). The hip joint of an operated fetus at E18.5 (Kihara et al., 1998) (g, h). (b, d, f, h) are magnified views of (a, c, e, g), respectively. Asterisks indicate the regions which are magnified. Scale bars are 100 μm.
operated neonates at birth (0.94 mm), and larger than the non-operated side FH of IV (0.94 mm).

In the observation of the neonates which survived for 12 hours, we detected roughness and deformity in the FH but could not detect any morphological difference from the operated side FH at birth by LM (in Table 2, data not shown). SEM observation of the FH surface could not detect a remarkable difference from that of the operated side FH at birth either (II, data not shown).

In the LM of the neonate that survived for 20 hours (III), apparent deformity or roughness was not detected in the operated side FH (Fig. 4a). One layer of squamous cells covered a part of the FH surface and the space between the underlying chondrocytes was wider than that of the operated neonates at birth (Fig. 4b vs. 2d). The operated side FH surface of IV (45 hours survival) was much smoother than that of the operated neonates at birth (Fig. 4c vs. 2c). One or two layers of flat squamous cells existed underneath the surface and the morphological features of the surface region became similar to those of the normal neonates (Fig. 4d vs. 2b). The surface of the non-operated side FH of IV looked as smooth as that of the operated side FH at a low magnification (Fig. 4e vs. 4c).

At a higher powered view, one thin cell layer covered the non-operated FH and one or two layers of flat squamous cells existed underneath the surface of the FH (Fig. 4f). The morphological features of the surface region of the operated side FH became similar to those of the non-operated side FH in IV, although the cell distance tended to be larger than that in the non-operated side (Fig. 4d vs. 4f).

DISCUSSION

CDH in children is an important disease to recognize because if it is left untreated it results in hip dysplasia (Walker, 1983). The etiology of CDH remains unclear, but it may be related to prenatal factors including intrauterine malposition (breech) (Luterkort and Marsal, 1985; Sival et al., 1993), multiple pregnancy, oligoamnios (Sival et al., 1990), ligamentous laxity attributable to either physiologic effects of hormones or a genetic trait (Wilkinson, 1992), and collagen disorder (Silence, 1992), as well as postnatal factors such as abnormal anatomic alignment of the hip, and extended or adducted positioning of the hip (Wilkinson, 1963). It has been suggested that some prenatal factors of CDH are related to the disturbance of intrauterine movements, especially those of the lower limbs (Silence, 1992). Several studies using postnatal young rabbits and rats reported that the experimental dysplasia of the hip joint was caused by fixation of the hind limbs and the wedge-shaped resection of the acetabulum (Wilkinson, 1963; Sijbrandji, 1965; Eronen et al., 1978; Soini and Ritsila, 1984). However, these experiments were only able to examine the disturbance of movements of the hind limbs as an etiology of dislocation of the hip during the post-weaning period, and the causes of dislocation of the hip during prenatal and weaning periods remain unknown.

In our previous study (Kihara et al., 1998), we examined the involvement of the fetal movements in the prenatal development of the hip joint. We compared the development of the hip joint in E18.5 fetuses that were operated on to restrict the motion of the hind limb on one side using an exo utero technique with those of non-operated fetuses, sham-operated fetuses and the non-operated side hip joint of the operated fe-
tuses as controls. Whereas gross development of the hip joint was not affected by the disturbed fetal movements, the surface of the FH of the operated fetuses was more irregular than that of the controls. Furthermore, the subsurface collagen fibers in the operated side FH at E18.5 remained as a random meshwork albeit finer than at the E17.5 controls, but did not form bundle- or sheet-like structures which were parallel to the joint surface as in the E18.5 controls. Although disturbed fetal movements appeared to delay the maturity of the chondrocytes and the matrix in the surface of the FH and acetalbum, cell shape and arrangement as well as collagen fiber structure were not simply delayed but, more correctly, abnormal. These results suggest that the proper development and maturation of the hip joint occur by cooperative effects between the development driven by genetic programs and appropriate modification at least partially by mechanical stress due to fetal movements. These previous studies which focused on the post-weaning period (Sijbrands, 1965; Soini and Ritsila, 1984) and our previous study which examined the late pregnancy period (E16.5-E18.5) are not sufficient to fully reveal the etiology of CDH, especially to analyze the relation between prenatal and postnatal factors, because the perinatal period remained unexamined. Therefore, we first aimed in this study to experimentally examine the effect of the restraint of fetal movement on the development of the hip joint until term (E22.5). We further studied the morphological change of the hip joint after releasing the restraint from the neonates in order to clarify the effect of postnatal joint movement on the abnormalities of the FH induced by the restriction of fetal movement.

BW of the neonates operated on at E16.5 and developed *exo utero* until E22.5 was significantly less than that of the neonates developed normally in utero, in contrast to the result at E18.5 without a significant difference between the operated and non-operated fetuses (Kihara et al., 1998). However, the LDFH of the operated side FH did not differ significantly from that of the normal or from non-operated side FH in the operated neonates at E22.5. The LDFH of the operated neonates at E22.5 (0.84 ± 0.05 mm) was much larger than that of the operated fetuses at E18.5 (0.49 ± 0.05 mm), as we reported previously (Kihara et al., 1998), and was not significantly different from the controls at E22.5 (Table 1). These results indicate that although *exo utero* development for 6 days till the term affected the weight gain possibly due to a delay in gaining weight of the soft tissues, the gross bone development *exo utero* was relatively well preserved.

In contrast, the shape of the FH of the neonate operated on at E16.5 and observed at E22.5 was deformed and the surface was more irregular than that of controls. Whereas we could not detect the deformity of the FH caused by restraint at E18.5 in LM observation at a low magnification (Kihara et al., 1998), the 6-days-restraint induced an obvious deformity of the FH at E22.5 (Figs. 2c and 2d). In SEM observation, the surface of FH of the operated side at E22.5 was rough and many mounds which corresponded to the nuclei of the mesenchymal cells on the FH surface were seen on the surface (Fig.
3b). These results suggested that the restraint of fetal movement for 6 days disturbed the flattening of the mesenchymal cells to eventually cause deformity of the FH surface in the operated neonates. However, it appeared that the gross enlargement of the hip joint did not delay compared to the control, suggesting that the basic cellular activities such as secretion and proliferation of chondrocytes remained intact.

We bred the operated neonates artificially in order to analyze the effects of the postnatal joint movement on the abnormalities of the FH caused by the restriction of prenatal movement and obtained 4 neonates which survived for a considerable period (Table 2). By LM, there was no difference in the surface of operated side FH between the operated neonate bred postnatally for 12 hours and the operated neonates at E22.5. However, the operated neonate bred for 45 hours showed an increased LDHF and the surface of the operated side FH was smoother and the cell arrangement was normalized compared with the operated sided FH of the neonates at E22.5. It was thus suggested that the abnormalities of the FH surface caused by the restriction of fetal movements of the hind limbs were significantly repaired by the limb movement after birth.

We did not observe any apparent dislocation or subluxation of the hip joint in the present operated neonates. This may indicate that the prenatal disturbance of movement of the hind limb alone does not cause overt CDH. In addition to the environmental factors, genetic factors are included in the etiology of many cases of CDH, as deduced from twin studies (Carter and Wilkinson, 1964; Wilkinson, 1992). No significant genetic predisposition for CDH has been documented for Wistar rats used for the present study. Therefore, the present experimental system serves as an animal model which has a prenatal environmental factor but not a genetic predisposition. By combining with the use of genetically predisposed animals (Lust et al., 1980), this system may serve to elucidate the relationship between genetic and prenatal environmental factors in the etiology of CDH. By additionally combining with postnatal mechanical stress, we will be able to examine the relationship between prenatal and postnatal etiologies and the effect of postnatal treatments for prenatal genetic and/or environmental etiology-based CDH.

In conclusion, we showed that the restraint of the hind limb of rat fetuses during pregnancy caused deformity of the hip joint at birth. We also demonstrated the process of the postnatal repair of the abnormalities induced by the restriction of fetal movement. These results suggest that the proper movement of the extremities prevents the CDH by repairing immaturity of the hip joint caused by prenatal environmental factors, and that the operated fetuses in the present ex utero system may be useful in investigating the involvement of mechanical stress as prenatal etiology of CDH of non-genetic type in relation to prenatal genetic and/or postnatal etiology-based CDH.

REFERENCES


Sival DA, Visser GHA, Precht HFR (1990) Does reduction


