TITLE PAGE

Title:

Compromised vitality of spermatozoa after contact with colonic mucosa in mice. Implications for fertility in colon vaginoplasty patients.

Authors:

Seitaro Kosaka[1], Masahiro Takeda[1], Takanori Ochi[1], Katsumi Miyahara[1], Eri Nakamura[2], Norihiro Tada[2], Geoffrey J. Lane[1], Atsuyuki Yamataka[1]

Institution:

[1]Department of Pediatric General and Urogenital Surgery,
Juntendo University School of Medicine, Tokyo, Japan

[2]Research Institute for Diseases of Old Age,
Juntendo University Graduate School of Medicine, Tokyo, Japan

Correspondence and address reprint request to:

Seitaro Kosaka

Department of Pediatric General and Urogenital Surgery,

Juntendo University School of Medicine, Tokyo, Japan

2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

Tel: +81-3-3813-3111

Fax: +81-3-5802-2033

E-mail: s-kosaka@juntendo.ac.jp

Abstract

Aim of the Study: Colon vaginoplasty (CV) is often performed for cloacal malformation (CM). We used mice to study the vitality of spermatozoa after contact with colonic mucosa as a factor contributing to infertility.

Methods: Spermatozoa isolated from the epididymides of C57BL/6J male mice (n=23) were syringed directly into the vaginas (Vag-group) or colons (Colo-group) of female mice (n=45). Vitality was determined by assessing motility by using Computer Assisted Sperm Analysis (CASA), viability by staining with SYBR-14 and propidium iodide, and fertility by in-vitro fertilization, prior to deposition, and at 5, 10, 30, and 60 minutes after deposition.

Main Results: Motility was significantly decreased in Colo only at 10 and 60 minutes. Viability of Colo spermatozoa was significantly at all assessment times, except at 10 minutes. Normal fertilization was observed with all Vag spermatozoa, but with Colo, there was arrest of embryo development with spermatozoa collected at 5 and 10 minutes, and no fertilization with spermatozoa collected at 30 and 60 minutes.

Conclusions: The vitality of spermatozoa is compromised by contact with colonic mucosa which could contribute to infertility in CM after CV because their ovaries and fallopian tubes are considered to be normal.

KEY WORDS: cloacal malformation, colon vaginoplasty, spermatozoa, infertility

Introduction

Cloacal malformation (CM), defined as confluence of the rectum, vagina, and urethra into a single common channel, is rare; reported incidences range from 1: 20,000-50,000 live births [1]. CM is treated by posterior sagittal anorectoplasty (PSARP) and total urogenital mobilization (TUM). The severity of the vaginal defect directly influences the choice of procedure for vaginal reconstruction, and multiple surgical techniques have been described which fall into one of 3 categories; skin-flap vaginoplasty, or pull-through vaginoplasty. In CM with a common channel longer than 3cm, vaginal replacement is usually required, and colon is the most common choice (colon vaginoplasty; CV) [2].

However, there is a scarcity of reports on long-term follow-up and quality of life in CM and reports concerning fertility and pregnancies in patients with CV are largely lacking in the literature. In fact, despite reportedly normal ovaries and fallopian tubes in CM, none of our cases has reported pregnancy to the best our knowledge.

We studied the vitality of spermatozoa, i.e., their motility, viability, and fertility, before and after exposure to colonic mucosa in mice, as a contributing factor to infertility after CV.

Materials and Methods

Spermatozoa suspension

The cauda epididymides of 10-week-old C57BL/6J male mice (n=23) were removed and incised with a pair of micro-spring scissors and clots of spermatozoa obtained by gentle milking with forceps. Harvested spermatozoa were placed in 15μL of TYH medium and incubated at 36℃ [3, 4] to make a spermatozoa suspension that was deposited directly into the vaginas (Vag-group) or the colons per ani (Colo-group) of 9-week-old C57BL/6J female mice (n=45) using a syringe. Each mouse received 15μL of suspension. To optimize contact between the suspension sample and the vaginas and colons of recipient female mice, the vaginas and colons were washed with TYH medium before deposition. In addition, in the Colo-group, recipient female mice were fasted for 24 hours prior to deposition to prevent fecal contamination. Deposited suspension samples were collected in 20μL of injected PB1 [5] after being in contact with colonic mucosa for 5, 10, 30, and 60 minutes. These test samples were assessed

for overall vitality, motility, viability, and fertility as described below.

Overall vitality of spermatozoa in collected suspension samples

Motility was assessed using light microscopy. The concentration of spermatozoa was calculated by counting the number of spermatozoa with a disposable hemocytometer (C-Chip; NanoEnTek Inc., Seoul, Korea). C-Chip consists of 9 large squares, each measuring 1x1mm, and four corner squares are divided into 16 small squares. The number of spermatozoa in each of the 16 small squares at each corner were counted manually.

Motility: Computer Assisted Sperm Analysis (CASA) system: IVOS®II

Motility of spermatozoa in test samples was also assessed by using a Computer Assisted Sperm Analysis system (CASA, IVOS®II Hamilton-Thorne Research, Beverly, MA, USA) [6]. A 30μL sample of spermatozoa suspension diluted with PB1 was placed on a cell counting chamber (Leja^{R} standard counting chamber). The percentage of motile spermatozoa, classified as those moving at a velocity of 5μm/s in any direction, were calculated.

Viability: SYBR-14 and propidium iodide

The proportion of viable spermatozoa in test samples was assessed by means of a dual staining technique using the stains SYBR-14 and propidium iodide. SYBR-14, which permeates intact plasma membranes, causes viable spermatozoa to fluoresce green. Propidium iodide, which permeates only damaged plasma membrane, causes nonviable spermatozoa to fluoresce red [7]. Deposited suspension samples collected in 20μL of injected PB1 from vaginas and colons (test samples) were diluted to 45μL with PB1, 5μL of 50-fold dilution of SYBR-14 in PB1 was added and incubated at 37℃ for 5 minutes followed by 5μL of propidium iodide and incubated again at 37℃ for 5 minutes. After staining, 10μL of test spermatozoa solution was placed on a C-Chip for manual cell counting. The percentage of viable spermatozoa was calculated under fluorescence stereomicroscopy equipped with filter sets.

Fertility: In-vitro fertilization

C57BL/6J female mice superovulated with 10IU of pregnant mare's serum gonadotropin (PMSG) (Peamex; Sankyo Lifetech Inc., Tokyo, Japan) followed by 10IU of human chorionic gonadotropin (hCG) (Sigma-Alfrich; Missouri) approximately 48 hours later. Mice were sacrificed 14 hours after the hCG injection for collection of oocytes. Cumulus-oocyte-complexes (COC) were released by opening the ampulla of

the oviduct with a dissecting needle. COC were transferred to a dish in a drop of TYH medium and 25μL of spermatozoa suspension diluted with PB1 was added. Each dish was placed in an incubator (37℃ 5% CO2 in air). 5 hours after insemination, the oocytes were washed in fresh TYH medium in a washing dish to remove any parthenogenetic oocytes. Dishes were observed for pronuclear stage eggs, i.e., fertilized eggs, 6 hours after insemination. Developmental stages used for classification were 2-cell, 4-cell, morula, blastocyst, and hatched blastocyst (Fig. 1). The percentages of pronuclear stage eggs were calculated. Subsequent development was monitored and the percentage of embryos at each stage was calculated.

Fig. 1

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical evaluation of differences between Vag and Colo was performed using the Chi-squared test for unpaired comparisons using JMP® 14 (SAS Institute Inc., Cary, NC, USA). Statistical significance was defined as $P<0.05$.

Results

All spermatozoa suspensions collected from Vag and Colo mice contained spermatozoa in concentrations ranging from 1-5x10^5/mL, which was comparable to concentrations of spermatozoa obtained from normal C57BL/6 epididymides.

Motility

The mean proportion of motile spermatozoa prior to deposition was 39.4±1.7%. While motility decreased over time in both Vag and Colo, motility in Colo was significantly lower at 10 and 60 minutes ($p<0.05$). Differences were not statistically significant at 5 and 30 minutes ($p=0.14$, 0.77, respectively). The decrease in motility was greater and more rapid in Colo, plummeting to only 0.8±0.3% at 60 minutes compared with a more gradual decrease in Vag from 10.0±2.7% at 10 minutes to 6.0±1.6% at 60 minutes (Fig. 2).

Fig. 2

Viability

Mean viability prior to deposition was 62.0±3.4%. While viability decreased over time in both Vag and Colo, viability in Colo was significantly lower at all assessment times except in suspensions collected after 10 minutes ($p < 0.05$). While viability in Vag basically did not change from 5 to 60 minutes [31.2±2.8%-38.9±5.5%], viability in Colo decreased over time, and was only 10.8±2.0% at 60 minutes (Fig. 3).

Fig. 3

Fertility

Maximum fertility in mice would appear to require concentrations of spermatozoa of 10^5-10^7/mL [8]. Fertility with spermatozoa obtained directly from the epididymides of mice was 31.0±3.4%. Normal fertilization was observed with all Vag spermatozoa, with development observed to the hatched blastocyst stage. In contrast, while normal fertilization was observed with Colo spermatozoa that was collected after contact with colonic mucosa for 5 and 10 minutes, there was arrest of embryo development after fertilization to blastocyst and morula stages, respectively, and with Colo spermatozoa that was collected after contact with colonic mucosa for 30 and 60 minutes, there was no fertilization at all. Development rates to the 4-cell, morula, blastocyst, and hatched blastocyst stages with 5 minute Colo spermatozoa (45.0%, 13.3%, 13.3%, and 0%) were lower than for 5 minute Vag spermatozoa (71.1%, 68.7%, 68.7%, 44.4%, respectively), and were also lower with 10 minute Colo spermatozoa (58.3%, 50.0%, 0%, and 0%, respectively) compared with 10 minute Vag spermatozoa (72.0%, 72.0%, 67.0%, 32.0%, respectively).

Discussion

All CM have formidably challenging urologic/gynecologic/anorectal anomalies that will profoundly influence their entire life. As surgery becomes less invasive and scars more cosmetic, functional issues, particularly those related to sexual and reproductive function become relevant as CM patients marry and desire to have children like other women. Unfortunately, for the majority of pediatric surgeons, the aims of vaginal reconstruction are to provide a cosmetically satisfactory introitus, a conduit for normal menstruation and pain-free penetrative intercourse. In other words structural integrity has priority over function, and fertility is not of concern to many surgeons planning vaginal reconstruction.

We could not identify any reports in the English language literature on whether there is a causal relationship between vaginal reconstruction and infertility in CM. Nevertheless, here, we report for the first time, a definite detrimental effect of colonic mucosa on the vitality of spermatozoa (in mice) which could be implicated as a cause of infertility in CV patients if there is in fact more infertility in CV patients.

As girls with CM grow, there may be accumulation of menstrual blood secondary to obstruction at puberty which causes severe abdominal pain with monthly exacerbations

and potential for retrograde flow of menstrual blood into the peritoneal cavity. Fallopian tube damage or blockage is often caused by inflammation caused by such backflow which can also cause pelvic adhesions that can be as severe as the bands of scar tissue that may form after abdominal or pelvic surgery and bind organs.

Causes of female infertility may include: uterine or cervical abnormalities, fallopian tube damage or blockage, postoperative pelvic adhesion, ovulation disorders, endometriosis or primary ovarian insufficiency or other specific or multiple physical and physiological factors, including genetic alterations [9]. Aberrant epigenetic mechanisms have also be associated with reproductive infertility. Approximately 40% of CM patients have duplicated Müllerian systems with two vaginas and 2 uteri [10]. Uterine duplication affects reproductive function, with a pooled spontaneous miscarriage rate of 32.2%, a pre-term birth rate of 28.3%, a term delivery rate of 36.2%, and a live birth rate of 55.9% [11]. In the report of Rackow et al., uterine anomalies were associated with difficulty in maintaining a pregnancy but not an impaired ability to conceive [12].

Although there are many factors that might contribute to infertility and recurrent pregnancy loss, several authors have reported good reproductive outcomes in CM. Actually, of Hendren's [13] 24 adult patients, seventeen have coitus, six had gone successfully through pregnancies. Five of these delivered by cesarean section and one vaginally. In the report Warne et al., there have been no pregnancies to date in their series [14]. In the report of Couchman et al., eight of 19 patients were reported to be sexually active, of whom 1 identified difficult penetration [15]. Three of the eight patients with sexual activity were attempting to conceive with assisted conception methods and one patients had a complex preterm delivery. Of the Rintala et al., 14 of 27 patients were reported to be sexually active [16]. Three had delivered healthy babies by cesarean section. In the report of Salvi et al., CM with long common channel had delivered the baby by cesarean section, but the detail of vaginal reconstruction is not described [17].

Complications associated with CV include vaginal stenosis. Strictures occur at the junctional zone between the intestine and perineal skin. All the dilatations were needed for strictures with routine home dilatation. Patients occasionally have painful intercourse due to a scar neuroma at the vaginal introitus. The rates of sexual activity in CV patients may be less than in other females.

While our study introduces new and important information about compromised vitality of spermatozoa after contact with colonic mucosa, any infertility in CV patients is actually treated conventionally by using in-vitro fertilization and blastocyst transfer [18].

Conclusion

Irrespective of the indications for CV, it is a procedure that is performed at a reasonable enough frequency for our findings to be relevant to the quality of life of postoperative CV patients and by inference, CM patients. In other words, fertility issues may be related to the compromised vitality of spermatozoa seen after contact with colonic mucosa that we observed in mice in this study. However, we can offer no explanation for our findings; why should contact with colonic mucosa compromise vitality of spermatozoa? Is pH involved, or some aspect of mucus quality? Further investigation is necessary to clarify the etiologic factors involved.

References

1. Versteegh HP, van Rooij IA, Levitt MA, Sloots CE, Wijnen RM, de Blaauw I (2013) Long-term follow-up of functional outcome in patients with a cloacal malformation: a systematic review. J Pediatr Surg 48:2343-2350
2. Pena A, Levitt MA, Hong A, Midulla P (2004) Surgical management of cloacal malformations: a review of 339 patients. J Pediatr Surg 39:470-479
3. Toyoda Y, Yokoyama M, Hoshi T (1971) Studies on the fertilization of mouse eggs in vitro. I. In vitro fertilization of eggs by fresh epididymal sperm. Jap J Anim Reprod 16:147-151.
4. Liu L, Nutter LM, Law N, McKerlie C (2009) Sperm freezing and in vitro fertilization in three substrains of C57BL/6 mice. J Am Assoc Lab Anim Sci 48:39-43
5. Tateno H, Kamiguchi Y (2007) Evaluation of chromosomal risk following intracytoplasmic sperm injection in the mouse. Biol Reprod 77:336-342
6. Itach SB, Finklestein M, Etkovitz N, Breitbart H (2012) Hyper-activated motility in sperm capacitation is mediated by phospholipase D-dependent actin polymerization. Dev Biol 362:154-161
7. Garner DL, Johnson LA (1995) Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 53:276-284
8. Lynn R. Fraser, Maudlin I (1978) Relationship between sperm concentration and the incidence of polyspermy in mouse embryos fertilized in vitro. J ReprodFert 52:103-106
9. Rodgers AB, Morgan CP, Leu NA, Bale TL (2015) Transgenerational epigenetic programming via sperm microRNA recapitulates effects of paternal stress. Proc Natl Acad Sci U S A 112:13699-13704
10. Levitt MA, Pena A (2010) Cloacal malformations: lessons learned from 490 cases. Semin Pediatr Surg 19:128-138
11. Grimbizis GF, Camus M, Tarlatzis BC, Bontis JN, Devroey P (2001) Clinical implications of uterine malformations and hysteroscopic treatment results. Hum Reprod Update 7:161-174
12. Rackow BW, Arici A (2007) Reproductive performance of women with mullerian anomalies. Curr Opin Obstet Gynecol 19:229-237
13. Hendren WH (1998) Cloaca, the most severe degree of imperforate anus: experience

with 195 cases. Ann Surg 228:331-346

14. Warne SA, Wilcox DT, Creighton S, Ransley PG (2003) Long-term gynecological outcome of patients with persistent cloaca. J Urol 170:1493-1496
15. Couchman A, Creighton SM, Wood D (2015) Adolescent and adult outcomes in women following childhood vaginal reconstruction for cloacal anomaly. J Urol 193:1819-1822
16. Rintala RJ (2016) Congenital cloaca: Long-term follow-up results with emphasis on outcomes beyond childhood. Semin Pediatr Surg 25:112-116
17. Salvi N, Arthur I (2008) A case of successful pregnancy outcome in a patient born with cloacal malformation. J Obstet Gynaecol 28:343-345
18. Papanikolaou EG, Camus M, Kolibianakis EM, Van Landuyt L, Van Steirteghem A, Devroey P (2006) In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. N Engl J Med 354:1139-1146

FIGURE LEGENDS

Fig. 1

Pronuclear stage eggs were classified as fertilized eggs. After fertilization, progress of development was classified according to the 4-cell, morula, blastocyst, and hatched blastocyst stages.

Fig. 2

Motility decreased from a pre-deposition value of 39.4±1.7% in both Vag and Colo, but was significantly lower in Colo only after 10 and 60 minutes ($p < 0.05$).

Fig. 3

Viability deceased from a mean pre-deposition value of 62.0±3.4% in both Vag and Colo. Viability in Colo was significantly lower at each assessment time except at 10 minutes ($p < 0.05$).

Fig.1

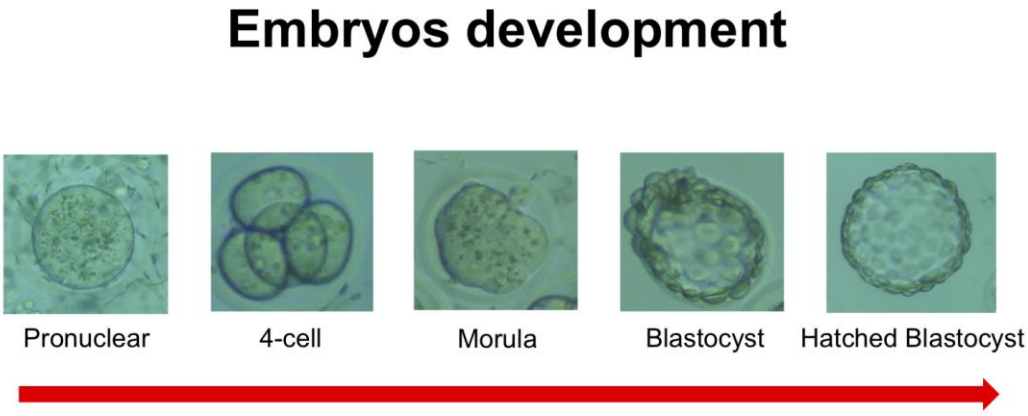

Embryos development
Pronuclear
4-cell
Morula
Blastocyst
Hatched Blastocyst

Fig.2

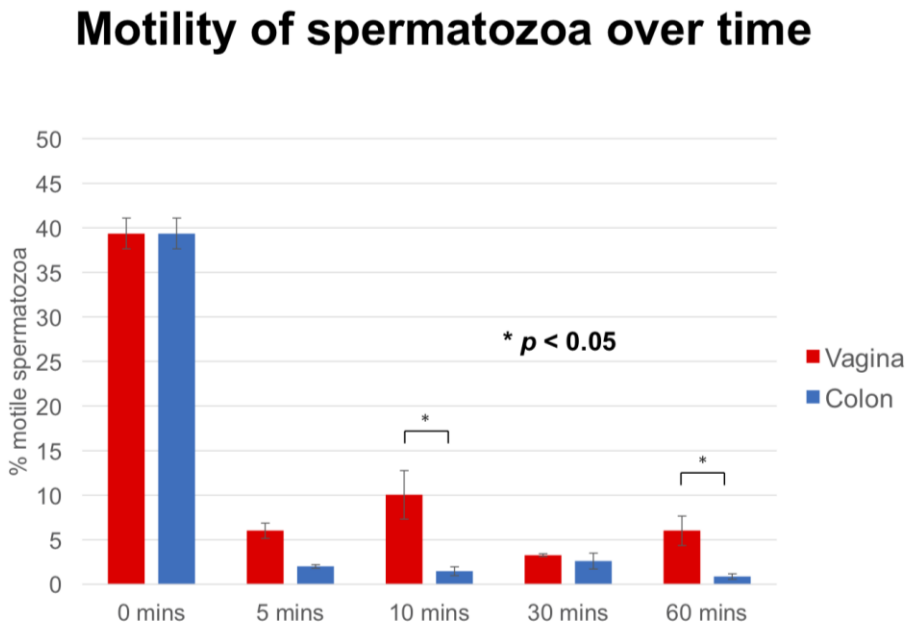
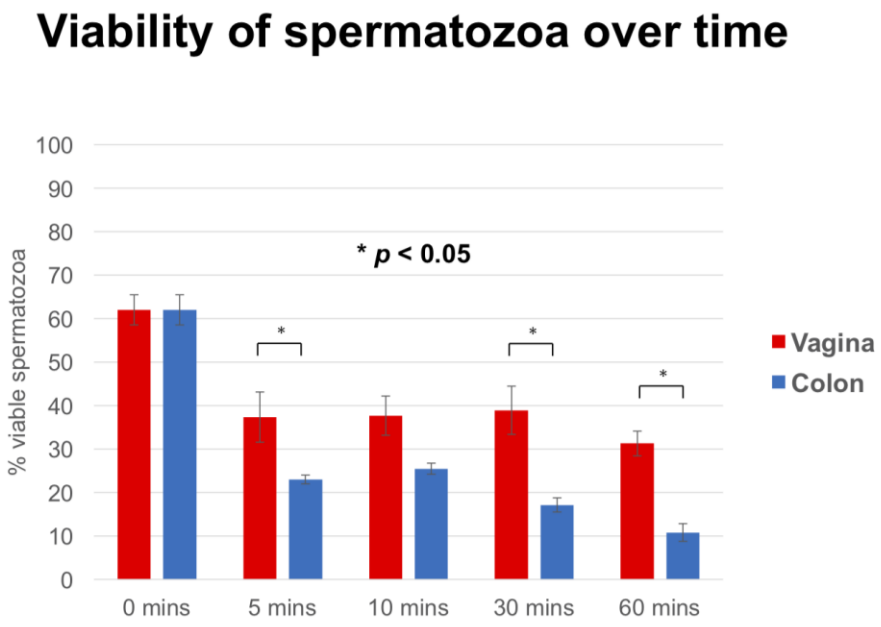

Motility of spermatozoa over time
% motile spermatozoa
50
45
40
35
30
25
20
15
10
5
0
* p < 0.05
*
*
0 mins
5 mins
10 mins
30 mins
60 mins
Vagina
Colon

Fig.3


Viability of spermatozoa over time
* p < 0.05
% viable spermatozoa
100
90
80
70
60
50
40
30
20
10
0
0 mins
5 mins
10 mins
30 mins
60 mins
Vagina
Colon