Myomectomy reduces endometrial T2 relaxation times

Magnetic resonance imaging was used to measure the endometrial T2 relaxation times of patients with infertility with fibroma. Although the location of fibromas did not influence the T2 relaxation times, we did observe a significant decrease in endometrial T2 relaxation times after myomectomy. (Fertil Steril® 2011;■:■–■. ©2011 by American Society for Reproductive Medicine.)

Key Words: MRI, T2 relaxation time, fibroid, myomectomy, endometrium

Although uterine fibroids, which occur in 20% to 50% of women, are the most common type of solid pelvic tumor (1), the relationship between fibroids and infertility is not well established (2, 3). It has been reported that myomectomy can increase the pregnancy rate for patients with infertility (4). However, the mechanisms by which this occurs are not well understood. Several theories have been proposed. First, it is possible that fibroids alter uterine cavity contour, resulting in mechanical pressure. Alternatively, the fibroids may induce abnormal uterine contractility (5, 6). Finally, local inflammation associated with the presence of fibroids may give rise to a hostile endometrial environment that impairs sperm transport and embryo implantation (5). It has been reported that excessive concentrations of inflammatory cytokines have deleterious effects on embryonic development and implantation (7, 8). Inagaki et al. (9) demonstrated that uterine cavities containing fibroids exhibit a state of excess inflammation, with up-regulation of matrix metalloproteinases and inflammatory cytokines such as interleukin-1 and tumor necrosis factor α.

Magnetic resonance imaging (MRI) is a high-resolution method of differentiating soft tissues. In MRI, the nuclei of atoms in samples first are aligned along a static magnetic field, then are excited to a higher-energy state by a radiofrequency signal, and then return to a lower-energy equilibrium state. T2 relaxation time is a parameter that describes the relaxation to the equilibrium state once the radiofrequency signal is turned off. As an assessment of inflammatory status, T2 relaxation time is a useful way to detect the inflammatory status of rheumatoid disease (10, 11), dermatomyositis (12), and Graves' orbitopathy in Graves' disease (13, 14). In the present study, we investigated the endometrial T2 relaxation times of patients with infertility with fibroma. We compared T2 relaxation times before and after surgery to examine the effect of myomectomy on the endometrium of patients with uterine fibroids.

A total of 35 patients with uterine fibroids who desire pregnancy were examined by MRI between September 2008 and October 2010 at Takinogawa Clinic. Inclusion criteria were as follows. First, patients had intramural- or submucosal-type fibroid. Second, in advance of MRI all patients underwent screening for ovulation and corpus luteum function. Patients had regular menstrual cycles of approximately 28 days. Basal levels of serum FSH, LH, and PRL on menstrual cycle day 3 through 5 were within normal range (criteria: FSH 3.5–12.5 mIU/mL, LH 2.4–12.6 mIU/mL, and PRL 4.9–29.3 ng/mL). Serum E_2 and P concentration in midluteal phase were >100 pg/mL and 10 ng/mL, respectively. After the screening test, ovarian functional status was monitored by basal body temperature (BBT) chart. An analysis was performed of BBT graphs, in which a rise in temperature of at least 0.2°C above that of the preceding 6 days that was completed in <48 hours and sustained for at least 11 days would indicate the occurrence of ovulation (15). All patients included in this study showed unequivocal biphasic cycles in their BBT chart. We designated the first day showing elevated temperature of at least 0.2°C as luteal phase day 1. Third, MRI was performed during the time of implantation window (luteal phase day 5–day 9), judged retrospectively by BBT chart (judged by gynecologists O.Y. and H.T.).

Osamu Yoshino, M.D., Ph.D.[a,b]
Masaaki Hori, M.D., Ph.D.[c]
Yutaka Osuga, M.D., Ph.D.[a]
Toshihiko Hayashi, M.D., Ph.D.[d]
Yoko Sadoshima, M.D.[b]
Hiroko Tsuchiya, M.D.[b]
Osamu Nishii, M.D., Ph.D.[b]
Yuji Taketani, M.D., Ph.D.[a]

[a] *Department of Obstetrics and Gynecology, University of Tokyo, Tokyo, Japan*
[b] *Department of Obstetrics and Gynecology, Mizonokuchi Hospital, Teikyo University, Kawasaki, Kanagawa, Japan*
[c] *Department of Radiology, Juntendo University, Tokyo, Japan*
[d] *Department of Radiology, Mizonokuchi Hospital, Teikyo University, Kawasaki, Kanagawa, Japan*

Received December 16, 2010; revised December 29, 2010; accepted January 7, 2011.
O.Y. has nothing to disclose. M.H. has nothing to disclose. Y.O. has nothing to disclose. T.H. has nothing to disclose. Y.S. has nothing to disclose. H.T. has nothing to disclose. O.N. has nothing to disclose. Y.T. has nothing to disclose.
Supported by Health and Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare of Japan and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology.
Reprint requests: Osamu Yoshino, M.D., Ph.D., and Yutaka Osuga, M.D., Ph.D., Department of Obstetrics and Gynecology, University of Tokyo, 7-1-3 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan (E-mail: oyoshino624@hotmail.co.jp; yutakaos-tky@umin.ac.jp).

Copyright ©2011 American Society for Reproductive Medicine, Published by Elsevier Inc.

By routine MRI study, the information retrieved included the location, number, and size of fibroids. Magnetic resonance studies were performed with use of a 1.5-T magnet unit (MRI machine from Siemens Japan, Shinagawa, Japan). Subsequently, conventional axial and sagittal T2–weighted images (repetition time [TR]/echo time [TE] = 4560–4720/107–111 milliseconds) and axial T1–weighted images (TR/TE = 550/8.5 milliseconds) were obtained with use of fast spin-echo techniques. T2 relaxation times of endometrium were measured on the same slice (350-mm field of view, 132 × 192 matrix, 3-mm slice thickness, bandwidth 362 Hz) with use of a spin-echo sequence. Eight images were acquired at each of the following TEs: 1.7, 23.4, 35.1, 46.8, 58.5, 70.2, 81.9, 93.6, 105.3, 117, and 128 milliseconds. The TR was 3 seconds, giving a total of 509 seconds acquisition time.

Ten out of 35 patients underwent myomectomy at Teikyo Mizonokuchi hospital. Among these 10 patients, 9 patients underwent laparoscopic-assisted myomectomy, and 1 patient underwent transcervical resection of fibroma. Four to 6 months after surgery, patients underwent a second MRI to evaluate T2 relaxation times during the implantation window. For statistical analysis, the Mann-Whitney *U* test was used for comparing between groups, and the paired *t*-test was used for comparing results before and after surgery.

T2 relaxation times in uterine endometrium obtained from patients with infertility who had intramural-type (n = 24) and submucosal-type (n = 12) fibroids were compared. We examined data from the midluteal phase. As shown in Figure 1A, the median value and minimum to maximum data of the two groups were 213 milliseconds (99–368 milliseconds) and 187 milliseconds (111–455 milliseconds) in intramural fibroids and submucosal fibroids, respectively. There was no statistical difference between groups (P=.9).

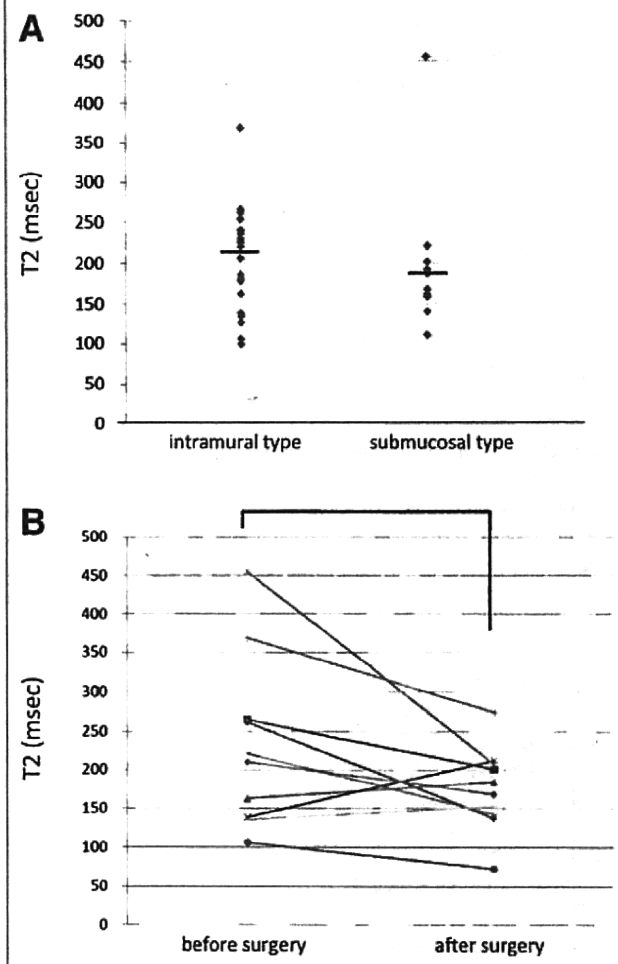
Because T2 relaxation times in the endometrium were comparable between intramural and submucosal fibroids (Fig. 1A), the data from both groups were combined in the subsequent study. After myomectomy, 10 patients underwent MRI at midluteal phase, and T2 relaxation times in the endometrium before and after surgery were compared. Of the 10 patients, 7 underwent surgery for intramural-type fibroids, and 3 underwent surgery for submucosal-type fibroids. As shown in Figure 1B, T2 relaxation times were decreased significantly after surgery (P=.03).

In the present study, we investigated the endometrial T2 relaxation times of patients with infertility with fibroma. We found that the endometrial T2 relaxation times were comparable regardless of the location of fibromas. Moreover, endometrial T2 relaxation times obtained after myomectomy were shortened significantly compared with the results before surgery.

Management of fibroids continues to present difficulties when used to treat infertility, because of a lack of understanding of the mechanisms by which fibroids impede pregnancy. Although myomectomy is recognized as a method to increase the rate of pregnancy (4), the precise mechanism of its contribution to fertility remains uncertain. It has been reported that the local inflammation associated with the presence of fibroids may result in a hostile endometrial environment that impairs fertility (7–9). Inagaki et al. (9) proved that the uterine cavities of patients with fibroids exhibited excessive inflammatory status. Accordingly, myomectomy might increase the fertility rate by decreasing the inflammatory

FIGURE 1

(A) T2 relaxation times in uterine endometrium obtained from patients with infertility who had intramural-type (n = 24) and submucosal-type (n = 12) fibroids were compared. The data from the midluteal phase were examined. (B) Ten patients underwent myomectomy and received MRI examination at the midluteal phase before and after surgery. T2 relaxation times in the endometrium were compared P=0.03.



Yoshino. Correspondence. Fertil Steril 2011.

status of the endometrium. In that study, 5 mL of saline solution was injected into the uterine cavity, and matrix metalloproteinase and cytokine levels of the fluid were measured to determine the inflammatory status directly (9). However, the volume of the uterine cavity can be decreased after removal of fibroids, making it difficult to compare precisely the inflammatory status before and after surgery. Therefore, it is necessary to develop less-invasive techniques that can estimate the inflammatory status of the uterine cavity. In the present study, we focused on T2 relaxation times obtained by MRI. This technique has proved useful in detecting the inflammatory activity of rheumatoid disease (10, 11), dermatomyositis (12), and Graves' orbitopathy in Graves' disease (13). Here, we observed a significant decrease in T2 relaxation times in patients examined after myomectomy. This suggests that myomectomy may suppress inflammatory activity in the endometrium.

T2 relaxation times in the human endometrium have been examined throughout the menstrual cycle. Varpula et al. (16) reported that a rapid increase in T2 relaxation times occurred during the proliferative phase, followed by little or no increase through the middle of the secretory phase. Hoad et al. (17) also reported that, during the periovulatory phase, T2 relaxation times were longer than in the other phases. They also observed that the variation in uterine tissue relaxation times between subjects was greater than the intrasubject cycle variation. Because of the large "normal" range, it might be very difficult to compare subjects or determine pathologic changes in the tissues from just a single measurement. However, because individuals exhibited similar increases and decreases over the menstrual cycle, the changes in T2 relaxation times within the same subject can be evaluated (17). Therefore, by comparing T2 relaxation times at the same menstrual phase obtained before and after myomectomy, the effect of surgery could be estimated. In our experiment, T2 relaxation times were measured during the "implantation window," the luteal phase day 5 to 9. We observed that there is no significant difference in T2 relaxation times between patients with fibroma and healthy volunteers (data not shown). Thus, measurement of T2 relaxation times would not be an effective way to detect uterine abnormalities, but it can be used to assess the success of myomectomy and is valuable in increasing our understanding of the pathophysiology of uterine fibroids in infertility. Other than inflammation (12, 14), iron content (18) is also known to increase T2 relaxation times. Therefore, further study is needed to confirm that T2 changes after myomectomy actually represent the change of inflammatory status in endometrium. This work represents a first step toward better understanding the relationship between T2 relaxation times and uterine fibroids in patients with infertility.

Acknowledgments: The authors thank Heather M. Martinez, Ph.D., for her helpful discussion and critical reading of the manuscript. They thank Yasufumi Shimizu, Ph.D., M.D., Kouji Motoyama, Ph.D., M.D., and Yasuhiro Kawamura, Ph.D., M.D. (Denentoshi Ladies' Clinic); Kenichi Tatsumi, Ph.D., M.D. (Umegaoka Women's Clinic); Susumu Tokuoka, Ph.D., M.D. (Tokuoka Women's Clinic); Ryo Matsuoka, Ph.D., M.D. (Tokyo Hitachi Hospital); and Takayoshi Ogawa, Ogawa, Ph.D., M.D. (Ogawa Clinic) for their supporting this study. They also thank Mr. Ryuji Nojiri and Mr. Yoshitsugu Funatsu (Takinogawa Clinic) for their technical assistance.

REFERENCES

1. Verkauf BS. Myomectomy for fertility enhancement and preservation. Fertil Steril 1992;58:1–15.
2. Donnez J, Jadoul P. What are the implications of myomas on fertility? A need for a debate? Hum Reprod 2002;17:1424–30.
3. Somigliana E, Vercellini P, Daguati R, Pasin R, De Giorgi O, Crosignani PG. Fibroids and female reproduction: a critical analysis of the evidence. Hum Reprod Update 2007;13:465–76.
4. Bulletti C, De Ziegler D, Polli V, Flamigni C. The role of leiomyomas in infertility. J Am Assoc Gynecol Laparosc 1999;6:441–5.
5. Richards PA, Richards PD, Tiltman AJ. The ultrastructure of fibromyomatous myometrium and its relationship to infertility. Hum Reprod Update 1998;4:520–5.
6. Yoshino O, Hayashi T, Osuga Y, Orisaka M, Asada H, Okuda S, et al. Decreased pregnancy rate is linked to abnormal uterine peristalsis caused by intramural fibroids. Hum Reprod 2010;25:2475–9.
7. Inoue T, Kanzaki H, Iwai M, Imai K, Narukawa S, Higuchi T, et al. Tumour necrosis factor alpha inhibits in-vitro decidualization of human endometrial stromal cells. Hum Reprod 1994;9:2411–7.
8. Kariya M, Kanzaki H, Takakura K, Imai K, Okamoto N, Emi N, et al. Interleukin-1 inhibits in vitro decidualization of human endometrial stromal cells. J Clin Endocrinol Metab 1991;73:1170–4.
9. Inagaki N, Ung L, Otani T, Wilkinson D, Lopata A. Uterine cavity matrix metalloproteinases and cytokines in patients with leiomyoma, adenomyosis or endometrial polyp. Eur J Obstet Gynecol Reprod Biol 2003;111:197–203.
10. Kight AC, Dardzinski BJ, Laor T, Graham TB. Magnetic resonance imaging evaluation of the effects of juvenile rheumatoid arthritis on distal femoral weight-bearing cartilage. Arthritis Rheum 2004;50:901–5.
11. Gasson J, Gandy SJ, Hutton CW, Jacoby RK, Summers IR, Vennart W. Magnetic resonance imaging of rheumatoid arthritis in metacarpophalangeal joints. Skeletal Radiol 2000;29:324–34.
12. Maillard SM, Jones R, Owens C, Pilkington C, Woo P, Wedderburn LR, et al. Quantitative assessment of MRI T2 relaxation time of thigh muscles in juvenile dermatomyositis. Rheumatology (Oxford) 2004;43:603–8.
13. Utech CI, Khatibnia U, Winter PF, Wulle KG. MR T2 relaxation time for the assessment of retrobulbar inflammation in Graves' ophthalmopathy. Thyroid 1995;5:185–93.
14. Hosten N, Sander B, Cordes M, Schubert CJ, Schomer W, Felix R. Graves ophthalmopathy: MR imaging of the orbits. Radiology 1989;172:759–62.
15. Ayres-de-Campos D, Silva-Carvalho JL, Oliveira C, Martins-da-Silva I, Silva-Carvalho J, Pereira-Leite L. Inter-observer agreement in analysis of basal body temperature graphs from infertile women. Hum Reprod 1995;10:2010–6.
16. Varpula M, Komu M, Irjala K. Relaxation time changes of the uterus during the menstrual cycle: correlation with hormonal status. Eur J Radiol 1993;16:90–4.
17. Hoad CL, Fulford J, Raine-Fenning NJ, Campbell BK, Johnson IR, Gowland PA. In vivo perfusion, T1, and T2 measurements in the female pelvis during the normal menstrual cycle: a feasibility study. J Magn Reson Imaging 2006;24:1350–6.
18. Argyropoulou MI, Metafratzi Z, Kiortsis DN, Bitsis S, Tsatsoulis A, Efremidis S. T2 relaxation rate as an index of pituitary iron overload in patients with beta-thalassemia major. AJR Am J Roentgenol 2000;175:1567–9.

特集
血管新生

卵胞発育と血管新生

吉野　修／大須賀　穣

Summary

卵胞周囲に血管内皮細胞が誘導されるのは二次卵胞後期であることが知られている。十分に発育した主席卵胞は豊富な血管網を有するのに対し，早期に発育が停止した閉鎖卵胞では疎な血管網が観察されることから，卵胞発育において卵胞周囲の良好な血管網発達が重要であることが知られている。血管の新生は血管内皮成長因子(VEGF)を代表とする血管新生因子により調整されているが，本稿では，特にその血管新生因子自体の制御について，卵胞発育の段階に絞って概説する。

Key words

卵胞発育●VEGF

サイトカイン●低酸素(hypoxia)

Osamu Yoshino

東京大学大学院医学系研究科産科婦人科学

帝京大学医学部附属溝口病院産婦人科

Yutaka Osuga

東京大学大学院医学系研究科産科婦人科学講師

はじめに

卵胞発育は，卵巣内外に存在する種々の因子により制御されているが，卵胞の発育段階により制御する因子が異なる。初期(原始，一次および二次卵胞の初期)の卵胞には，卵胞刺激ホルモン(FSH)受容体が発現しておらず，また卵胞周囲には血管のマーカーとなる血管内皮細胞の存在を認めない。すなわち，初期の卵胞発育においては，ゴナドトロピンを代表とする卵巣外から血流を介して卵胞へ到達する因子は関与せず，代わりにアクチビン，bone morphogenetic protein-15 (BMP-15) や growth differentiation factor-9 (GDF-9) などの卵胞内に存在する局所因子によりその制御を受けていることが考えられている。

卵胞がFSH受容体を獲得する二次卵胞後期以降になると，卵胞の発育は主にFSH依存性となる。このとき，FSHは下垂体から産生され，血流を介して卵胞に到達することから，FSH依存性となった卵胞がより多くのFSHの作用を受けるためには，卵胞周囲における血管網の発達が重要となることが予想される。事実，卵胞周囲に血管内皮細胞が誘導されるのは二次卵胞後期であることが知られており，十分に発育した主席卵胞は豊富な血管網を有するのに対し，早期に発育が停止した閉鎖卵胞では疎な血管網が観察される。

これまで，種々の臓器において血管の新生は血管新生因子により制御されていることが知られている。本稿では卵胞における血管網の発達を制御

する因子について，諸家により報告されている血管新生物質(VEGF，アンジオポイエチン)およびわれわれの研究グループのデータ(IL-8，アンジオゲニン，ミッドカイン)を取り上げる。

形　態

先に述べたように，原始卵胞周囲には血管内皮細胞は存在せず，二次卵胞の初期に莢膜細胞の増生がみられ，血管内皮細胞が誘導される[1]。その後，血管内皮細胞の増殖は続き，二次卵胞後期および三次卵胞において内莢膜細胞層にみられる増殖性細胞の25～30%を血管内皮細胞が占めている[1]。血管内皮細胞の増殖は莢膜細胞層に限定しており，顆粒膜細胞層では無血管野状態が続き，顆粒膜細胞はゴナドトロピン，栄養素，酸素の供給を莢膜細胞層から基底膜(basement membrane)を介した拡散により受け取る。このことから，莢膜細胞層における良好な血管網の発達が卵胞発育に重要な因子であることが推測される。実際，卵胞の増大に従って血流は増加し，特に主席卵胞では血流が著明に増加することが知られている[2]。

一方，閉鎖卵胞では莢膜細胞での血管網構築が不良であることから，血管新生誘導は単に卵胞発育のみに作用するのではなく，卵胞の選別にも関与しているのであろう。血管新生誘導に関して，これまで多くの血管新生因子の関与が報告されている。血管新生因子は顆粒膜細胞および莢膜細胞から産生され，その受容体は血管内皮細胞や血管周囲に存在するペリサイトに存在することが知られている。

低酸素環境と血管新生因子

卵胞発育中，血管新生は莢膜細胞層に限定しており，顆粒膜細胞層には無血管野状態が続く。排卵前，顆粒膜細胞は多層になっていることから，理論上，顆粒膜細胞は低酸素(hypoxia)に陥されると想定され[3]，事実，卵胞液中の酸素濃度は1%以下～5%との報告がある[4]。われわれも体外受精(IVF)症例の採卵時にヒト卵胞を個別に穿刺し，卵胞ごとの大きさと卵胞液中の溶存酸素濃度を調べたところ，卵胞が大きくなるに従い，卵胞液中の酸素濃度が低下することを認めた(図1)[5]。

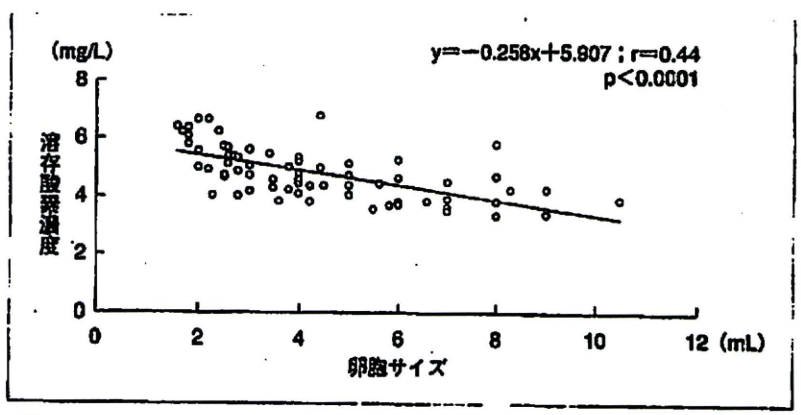


図1　ヒト卵胞の大きさと溶存酸素濃度の関係
IVFの採卵時に卵胞ごとの溶存酸素濃度を測定した。卵胞が大きくなるほど，溶存酸素が少ない(hypoxia)の傾向がみられた。
(文献5)より引用・改変)

種々の組織において，低酸素刺激が血管新生因子の誘導に関与していることが知られている。同様に，卵胞は体積の増大という構造的変化に伴う低酸素状態を利用して，血管新生因子を誘導している可能性が考えられる。血管新生因子を誘導する hypoxia-inducible factor 1α(HIF1α)の卵胞での発現を調べた検討では，HIF1αは排卵前の顆粒膜細胞に発現しており，また卵巣に血管内皮成長因子(vascular endothelial growth factor；VEGF)の阻害薬を投与することで卵胞が低酸素になる条件下では，HIF1αの発現が上昇することが報告されている[6]。

VEGF および EG-VEGF

1．VEGF

線維芽細胞成長因子(fibroblast growth factor；FGF)など多くの成長因子が種々の細胞に作用を有するのに対し，VEGF は血管内皮細胞に対してのみ特異的に作用することが知られている。VEGF のノックアウトマウスは血管が形成できないことで胎生致死に陥ることから，VEGF は血管形成に必須の物質である[7]。VEGF は，二次卵胞以降の顆粒膜細胞および莢膜細胞に発現することが知られている[8]。また，VEGF の受容体も二次卵胞周囲の血管内皮細胞から発現することが知られている。二次卵胞における VEGF の誘導因子に関してはまだ完全にはわかっていない。卵巣 VEGF の調節因子として，これまで FSH，黄体化ホルモン(LH)[9]が報告されているが，二次卵胞はゴナドトロピン受容体を有していないため，ゴナドトロピンによる VEGF 調節は考えにくい。また，VEGF の発現に重要な因子である HIF1α の卵巣における発現は，前胞状卵胞の段階では発現していないとの報告があり[6]，この時期には低酸素刺激による VEGF 誘導は考えにくいのかもしれない。その他，インターロイキン(IL)-1[10]や神経成長因子(nerve growth factor；NGF)[11]などによる VEGF 発現の報告もみられるが，いずれも排卵期の卵胞を想定した検討となっている。

VEGF 蛋白を卵巣周囲に直接投与することで，ラット[12]およびマウス[13]の卵胞数が上昇したことが報告されている。また，Shimizu らは VEGF 遺伝子をミニブタ卵巣に直接投与することで，卵胞液中の VEGF 蛋白質濃度の増加および莢膜細胞層に多くの血管新生が誘導されることを認めている[14]。さらに，同グループはラットを用いた検討において，*VEGF* 遺伝子投与により通常の過排卵処理に比べ排卵数が約 2 倍に増加すると報告している[15]。

VEGF の阻害実験として，サルに VEGF 阻害薬(a soluble decoy receptor-based inhibitor：VEGF trap)を投与した報告がある。図 2[16]に VEGF 受容体および VEGF trap の構造を示す。

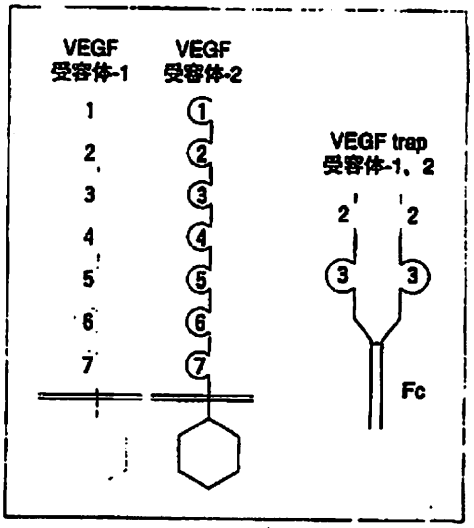


図2　VEGF 受容体-1，2および VEGF trap の構造
VEGF trap は VEGF 受容体-1，2の細胞外ドメインの一部を有しており，VEGF に結合しその作用を阻害する。
(文献16)より引用・改変)

VEGF trap は VEGF 受容体-1，2の細胞外ドメインの一部を有しており，強力な抗 VEGF 作用をもつ。現在，aflibercept として多くの癌種に対し臨床試験が進行中である。VEGF trap 投与により卵胞発育が阻害され，血中エストラジオール(E_2)およびインヒビンBのレベルも減少し，一過性に排卵が抑制された[16]-[18]。卵胞に対する効果は，特に大型胞状卵胞の発育が障害されていることがわかる(図3)[18]。興味深いことに，VEGF trap の投与により血管内皮細胞における VEGF 受容体-1，2も減少していた[16]ことから，VEGF 受容体はそのリガンドにより正に制御されている可能性がある。

これら VEGF 添加および阻害実験は，血管新生が卵胞発育に必須の因子であり，その調整に VEGF が大きく関わっていることを十分に支持するものであるが，多くは動物実験により得られた知見である。

ヒトにおいては，IVF 患者を用いた検討で，卵胞周囲の血管密度とその卵胞中 VEGF 濃度には正の相関を認め，また血管新生の発達した卵胞から得られた卵子は受精率および妊娠率が良好であったと報告されている[19]。また，IVF 患者の血清 VEGF 濃度測定により卵巣過剰刺激症候群(OHSS)の発症予測を検討した報告では，ヒト絨毛性ゴナドトロピン(hCG)投与日から採卵日までに VEGF 濃度上昇が過剰にみられた症例では，E_2値，卵胞数，卵子数よりも高感度に OHSS の発症を予測できたとしている[20]。

2．EG-VEGF

内分泌腺由来 VEGF(endocrine gland-derived VEGF；EG-VEGF)はホルモン産生臓器に存在する血管新生因子として注目されており[21]，プロキネチシン-1(PK-1)としても知られている。VEGF やアンジオポイエチンの受容体がチロシンキナーゼ型受容体であるのに対し，EG-VEGF の受容体は G 蛋白質共役受容体と報告されている[22]。ヒト卵巣において，EG-VEGF は原始卵胞や一次卵胞では顆粒膜細胞での発現が多いが，胞状卵胞になると莢膜細胞での発現が強くなり，顆粒膜細胞での発現は減るとの報告がある[23]。作用に関しては VEGF と同じと考えられているが，発現箇所およびその制御機構が異なることで，血管

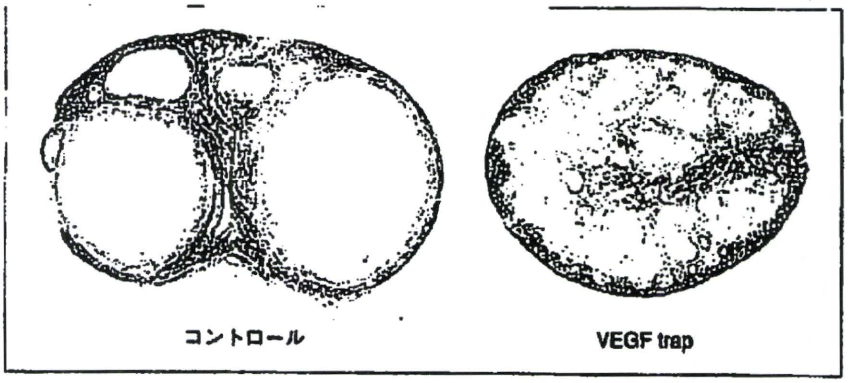


図3　VEGF trap の卵胞発育に対する効果
卵胞期に VEGF trap の投与を受けたマーモセット卵巣。VEGF trap 群は前胞状卵胞および小胞状卵胞を認めるが，大型の胞状卵胞はみられない。
(文献18)より引用)

新生に寄与していると考えられている。

二次卵胞から血管新生が誘導されてくる機序に比べて，初期卵胞が血管新生を誘導する機序はまだ不明な点が多い。二次卵胞からVEGFが発現することから，同分子の関与は十分に考えられるが，卵胞がはじめて血管を獲得することにVEGFが必須因子であるかに関しては不明である。その点，EG-VEGFは二次卵胞よりも初期の卵胞からその発現の報告があるため，卵胞への血管新生誘導に関与しているのかもしれない。

アンジオポイエチン-1，2

卵胞の血管は，卵胞期には短期間で成長・成熟し，閉鎖卵胞ではすぐに退縮するというユニークなパターンを示す。そこで血管の安定，脱安定に関与する因子であるアンジオポイエチン-1，2の関与が報告されている。

TIE2は血管内皮細胞や初期の血液細胞が有する血管新生，脈管形成に働くチロシンキナーゼ型受容体で，アンジオポイエチン-1はTIE2のリガンドとして同定された[24]。VEGFにより誘導された新生血管は未熟であるが，アンジオポイエチン-1はTIE2活性化を介して，ペリサイトを血管周囲に誘導させ，血管の安定化に寄与することが知られている[25]。実際，アンジオポイエチン-1のノックアウトマウスは，脈管形成の欠陥や出血により胎生致死に陥る。TIE2のノックアウトマウスも同様の表現系を呈する。

その後，ホモロジー検索により，アンジオポイエチン-2が同定された[26]。アンジオポイエチン-2はアンジオポイエチン-1によるTIE2リン酸化活性を抑制し，血管の脱安定化に作用する。多くの知見は黄体での検討がなされており，卵胞発育とアンジオポイエチンの論文は限られている。マウスを用いた検討ではアンジオポイエチン-1，2ともに主に莢膜細胞での発現を示している[26]。また，ラット卵巣を用いた検討でも，アンジオポイエチン-1，2およびTIE2の局在は莢膜細胞が主なものであり，卵胞発育につれアンジオポイエチン-1，2の発現が増加してくるのに対し，TIE2はどのステージにおいても強発現を認めたとしている[27]。Hazzardらはアカゲザルの顆粒膜細胞を用いた検討で，排卵期に顆粒膜細胞にアンジオポイエチン-1，2が発現することを報告している[28]。

ミッドカイン

ミッドカインは，ヘパリン結合増殖因子の1つであり，プレイオトロフィン(PTN)という物質に類似している。癌細胞などを用いた検討において，血管新生作用以外にも細胞遊走，細胞増殖など多くの作用を有することが知られている。卵胞液中にミッドカインおよびPTNが高濃度で存在すること，また興味深いことにミッドカインとPTN両因子のノックアウトマウスでは成熟卵胞の減少により不妊を呈することが報告されている[29]。

Ikedaらは，ミッドカインが体外成熟培養法(*in vitro* maturation；IVM)の系で卵子の発育を促進させること，またその機序としてミッドカインが直接卵子に作用するのではなく，卵子周囲に存在する顆粒膜細胞のアポトーシスを抑制することで卵子発育に働くことを示している[30]。

われわれは，ミッドカインがヒト顆粒膜細胞および莢膜細胞に発現し，ヒト顆粒膜細胞の増殖に作用することを明らかにしてきた[31]。これまでミッドカインは低酸素で誘導されることが知られており[32]，われわれの検討でもIVF患者の採卵時における卵胞ごとの溶存酸素濃度とミッドカインの濃度は逆相関していることから，低酸素刺激によるミッドカイン産生誘導が示唆された(図4)[31]。また，ミッドカインは卵胞発育の制御因子であるFSHやレチノイン酸により発現が促進されることが報告されている[33]。

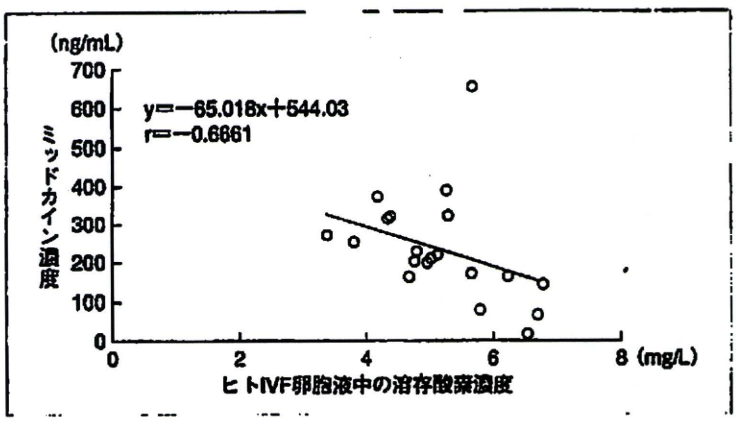


図4　ヒトIVF卵胞液中の溶存酸素とミッドカインの関係
卵胞の溶存酸素濃度とミッドカイン濃度は負の相関を示した。
(文献31)より引用・改変)

アンジオゲニン

アンジオゲニンは，当初大腸癌細胞の上清中より分離同定された物質である。種々の細胞より産生され，血管新生因子として作用することが知られている。Leeらは，ウシ卵巣を用いた検討で，アンジオゲニンが原始卵胞の顆粒膜細胞から認められ，その発現が卵胞の発育，黄体形成に従って増強することを示している[34]。

われわれのグループはアンジオゲニンがヒト卵胞液中に存在することを認め，ヒト顆粒膜細胞を用いた *in vitro* の系で，低酸素刺激により顆粒膜細胞からのアンジオゲニン産生が亢進することを認めた(図5)[35]。また，低酸素刺激以外にもhCGおよび環状アデノシン一リン酸(cAMP)刺激によりアンジオゲニンが誘導されることを見出している[35]。

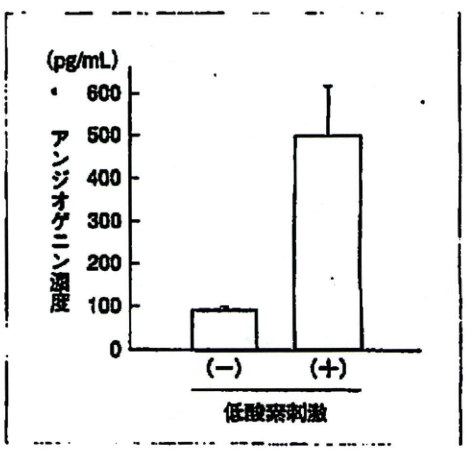


図5　ヒト顆粒膜細胞培養上清中のアンジオゲニン濃度
低酸素刺激により培養ヒト顆粒膜細胞からのアンジオゲニン産生が亢進した。
(文献35)より引用・改変)

IL-8

IL-8は，炎症性サイトカインであるとともに，血管新生因子であることが知られている[36]。また，好中球の遊走能をもつことから，排卵期にhCG刺激により誘導されるIL-8は，卵胞に炎症を惹起し排卵に関与することはよく知られている[37]。他の細胞において，低酸素刺激によりIL-8産生が誘導されることが報告されているが[38]，われわれの *in vivo* の検討でも，ミッドカイン同様にヒトIVF卵胞液中の溶存酸素濃度と

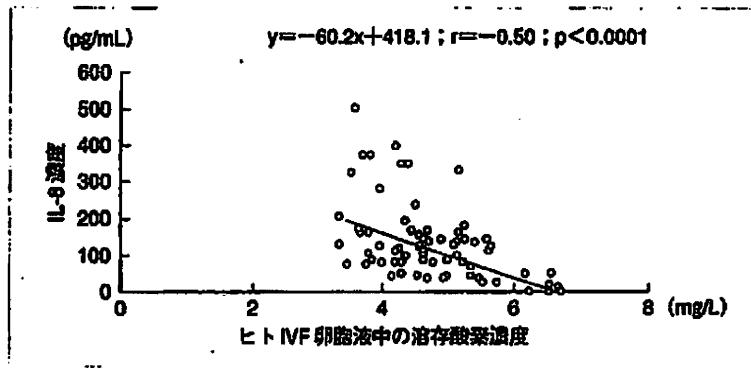


図6　ヒトIVF卵胞液中の溶存酸素とIL-8の関係
卵胞の溶存酸素濃度とIL-8濃度は負の相関を示した。
（文献5）より引用・改変）

IL-8濃度には負の相関を認めた（図6）[5][11]。

Changら[39]は，発育卵胞の顆粒膜細胞層にもIL-8が発現しており，好中球を莢膜細胞層に誘導していることを報告している。閉鎖卵胞のみでなく，良好な発育卵胞においても好中球の集積を認めているが，好中球の卵胞発育に対する作用はまだ不明である。また，ウサギにIL-8を投与したところ，成熟した胞状卵胞が誘導されることから，IL-8が卵胞発育に関与していることが示唆される[40]。

血管内皮細胞の血管透過性を調べた検討において，特にVEGFとIL-8が血管透過性に重要であったことから，両因子がOHSSの発症に深く関わっている可能性が報告されている。興味深いことに，IL-8はケモカイン受容体CXCR1/2の活性化を介して血管内皮細胞におけるVEGF受容体を誘導するとされている[41]。

おわりに

卵胞の血管新生は，血管関連細胞に特異的に作用する因子（VEGF，アンジオポイエチン）を中心に制御を受けていることは明らかであるが，顆粒膜細胞にも直接作用を有する他の血管新生因子によっても制御を受けていると思われる。血管新生因子のさらなる理解は，良好な卵胞発育のみならずOHSSなどの疾患制御にもつながる可能性がある。

文献

1）Wulff C, Wiegand SJ, Saunders PT, et al：Angiogenesis during follicular development in the primate and its inhibition by treatment with truncated Flt-1-Fc(vascular endothelial growth factor Trap(A40)). Endocrinology 142：3244-3254, 2001
2）Campbell S, Bourne TH, Waterstone J, et al：Transvaginal color blood flow imaging of the periovulatory follicle. Fertil Steril 60：433-438, 1993
3）Gosden RG, Byatt-Smith JG：Oxygen concentration gradient across the ovarian follicular epithelium；Model, predictions and implications. Hum Reprod 1：65-68, 1986
4）Van Blerkom J, Antczak M, Schrader R：The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid；Association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum Reprod 12：1047-1055, 1997

5) Yoshino O, Osuga Y, Koga K, et al : Upregulation of interleukin-8 by hypoxia in human ovaries. Am J Reprod Immunol 50 : 286-290, 2003
6) Duncan WC, van den Driesche S, Fraser HM : Inhibition of vascular endothelial growth factor in the primate ovary up-regulates hypoxia-inducible factor-1alpha in the follicle and corpus luteum. Endocrinology 149 : 3313-3320, 2008
7) Ferrara N, Carver-Moore K, Chen H, et al : Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380 : 439-442, 1996
8) Fraser HM, Wulff C : Angiogenesis in the primate ovary. Reprod Fertil Dev 13 : 557-566, 2001
9) Christenson LK, Stouffer RL : Follicle-stimulating hormone and luteinizing hormone/chorionic gonadotropin stimulation of vascular endothelial growth factor production by macaque granulosa cells from pre- and periovulatory follicles. J Clin Endocrinol Metab 82 : 2135-2142, 1997
10) Levitas E, Chamoun D, Udoff LC, et al : Periovulatory and interleukin-1 beta-dependent up-regulation of intraovarian vascular endothelial growth factor(VEGF) in the rat ; Potential role for VEGF in the promotion of periovulatory angiogenesis and vascular permeability. J Soc Gynecol Investig 7 : 51-60, 2000
11) Julio-Pieper M, Lozada P, Tapia V, et al : Nerve growth factor induces vascular endothelial growth factor expression in granulosa cells via a trkA receptor/mitogen-activated protein kinase-extracellularly regulated kinase 2-dependent pathway. J Clin Endocrinol Metab 94 : 3065-3071, 2009
12) Danforth DR, Arbogast LK, Ghosh S, et al : Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. Biol Reprod 68 : 1736-1741, 2003
13) Quintana R, Kopcow L, Sueldo C, et al : Direct injection of vascular endothelial growth factor into the ovary of mice promotes follicular development. Fertil Steril 82(Suppl3) : 1101-1105, 2004
14) Shimizu T, Jiang JY, Iijima K, et al : Induction of follicular development by direct single injection of vascular endothelial growth factor gene fragments into the ovary of miniature gilts. Biol Reprod 69 : 1388-1393, 2003
15) Shimizu T, Iijima K, Miyabayashi K, et al : Effect of direct ovarian injection of vascular endothelial growth factor gene fragments on follicular development in immature female rats. Reproduction 134 : 677-682, 2007
16) Wulff C, Wilson H, Wiegand SJ, et al : Prevention of thecal angiogenesis, antral follicular growth, and ovulation in the primate by treatment with vascular endothelial growth factor Trap R1R2. Endocrinology 143 : 2797-2807, 2002
17) Fraser HM, Wilson H, Rudge JS, et al : Single injections of vascular endothelial growth factor trap block ovulation in the macaque and produce a prolonged, dose-related suppression of ovarian function. J Clin Endocrinol Metab 90 : 1114-1122, 2005
18) Fraser HM, Duncan WC : Vascular morphogenesis in the primate ovary. Angiogenesis 8 : 101-116, 2005
19) Monteleone P, Giovanni Artini P, Simi G, et al : Follicular fluid VEGF levels directly correlate with perifollicular blood flow in normoresponder patients undergoing IVF. J Assist Reprod Genet 25 : 183-186, 2008
20) Agrawal R, Tan SL, Wild S, et al : Serum vascular endothelial growth factor concentrations in *in vitro* fertilization cycles predict the risk of ovarian hyperstimulation syndrome. Fertil Steril 71 : 287-293, 1999
21) LeCouter J, Kowalski J, Foster J, et al : Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 412 : 877-884, 2001
22) Kisliouk T, Levy N, Hurwitz A, et al : Presence and regulation of endocrine gland vascular endothelial growth factor/prokineticin-1 and its receptors in ovarian cells. J Clin Endocrinol Metab 88 : 3700-3707, 2003
23) Ferrara N, Frantz G, LeCouter J, et al : Differential expression of the angiogenic factor

genes vascular endothelial growth factor (VEGF) and endocrine gland-derived VEGF in normal and polycystic human ovaries. Am J Pathol 162 : 1881-1893, 2003

24) Davis S, Aldrich TH, Jones PF, et al : Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. Cell 87 : 1161-1169, 1996

25) Suri C, Jones PF, Patan S, et al : Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 87 : 1171-1180, 1996

26) Maisonpierre PC, Suri C, Jones PF, et al : Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. Science 277 : 55-60, 1997

27) Abramovich D, Rodriguez Celin A, Hernandez F, et al : Spatiotemporal analysis of the protein expression of angiogenic factors and their related receptors during folliculogenesis in rats with and without hormonal treatment. Reproduction 137 : 309-320, 2009

28) Hazzard TM, Molskness TA, Chaffin CL, et al : Vascular endothelial growth factor (VEGF) and angiopoietin regulation by gonadotrophin and steroids in macaque granulosa cells during the peri-ovulatory interval. Mol Hum Reprod 5 : 1115-1121, 1999

29) Muramatsu H, Zou P, Kurosawa N, et al : Female infertility in mice deficient in midkine and pleiotrophin, which form a distinct family of growth factors. Genes Cells 11 : 1405-1417, 2006

30) Ikeda S, Saeki K, Imai H, et al : Abilities of cumulus and granulosa cells to enhance the developmental competence of bovine oocytes during *in vitro* maturation period are promoted by midkine ; A possible implication of its apoptosis suppressing effects. Reproduction 132 : 549-557, 2006

31) Hirota Y, Osuga Y, Nose E, et al : The presence of midkine and its possible implication in human ovarian follicles. Am J Reprod Immunol 58 : 367-373, 2007

32) Reynolds PR, Mucenski ML, Le Cras TD, et al : Midkine is regulated by hypoxia and causes pulmonary vascular remodeling. J Biol Chem 279 : 37124-37132, 2004

33) Minegishi T, Karino S, Tano M, et al : Regulation of midkine messenger ribonucleic acid levels in cultured rat granulosa cells. Biochem Biophys Res Commun 229 : 799-805, 1996

34) Lee HS, Lee IS, Kang TC, et al : Angiogenin is involved in morphological changes and angiogenesis in the ovary. Biochem Biophys Res Commun 257 : 182-186, 1999

35) Koga K, Osuga Y, Tsutsumi O, et al : Evidence for the presence of angiogenin in human follicular fluid and the up-regulation of its production by human chorionic gonadotropin and hypoxia. J Clin Endocrinol Metab 85 : 3352-3355, 2000

36) Kitadai Y, Takahashi Y, Haruma K, et al : Transfection of interleukin-8 increases angiogenesis and tumorigenesis of human gastric carcinoma cells in nude mice. Br J Cancer 81 : 647-653, 1999

37) Ujioka T, Matsukawa A, Tanaka N, et al : Interleukin-8 as an essential factor in the human chorionic gonadotropin-induced rabbit ovulatory process ; Interleukin-8 induces neutrophil accumulation and activation in ovulation. Biol Reprod 58 : 526-530, 1998

38) Metinko AP, Kunkel SL, Standiford TJ, et al : Anoxia-hyperoxia induces monocyte-derived interleukin-8. J Clin Invest 90 : 791-798, 1992

39) Chang RJ, Gougeon A, Erickson GF : Evidence for a neutrophil-interleukin-8 system in human folliculogenesis. Am J Obstet Gynecol 178 : 650-657, 1998

40) Belayet HM, Kanayama N, Khatun S, et al : Pharmacologic doses of interleukin 8 suppositories induce follicular maturation in rabbits. Cytokine 12 : 361-367, 2000

41) Chen SU, Chou CH, Lin CW, et al : Signal mechanisms of vascular endothelial growth factor and interleukin-8 in ovarian hyperstimulation syndrome ; Dopamine targets their common pathways. Hum Reprod 25 : 757-767, 2010

Journal of Reproduction and Development, Vol. 56, No. 2, 2010

—Technology Report—

Viability of Porcine Embryos after Vitrification Using Water-soluble Pullulan Films

Nobutada SAKAGAMI[1)], Tadashi YAMAMOTO[1)], Kiyoshi AKIYAMA[1)], Yoshinori NAKAZAWA[1)], Nobuo KOJIMA[1)], Kouji NISHIDA[1)], Shoko YOKOMIZO[2)], Yuji TAKAGI[2)], Hiroyuki ABE[3)], Chie SUZUKI[4)] and Koji YOSHIOKA[4)]

[1)]Kanagawa Prefectural Livestock Industry Technology Center, Ebina 243-0417, [2)]Faculty of Agriculture, Shinshu University, Minamiminowa 399-4598, [3)]Graduate School of Science and Engineering, Yamagata University, Yonezawa 992-8510 and [4)]National Institute of Animal Health, Tsukuba 305-0856, Japan

Abstract. The efficiency of a porcine embryo vitrification method that uses water-soluble films of pullulan, a naturally-occurring polysaccharide polymer, was compared with two other types of vitrification methods using different devices and solutions for vitrification and warming. Blastocysts collected *in vivo* and vitrified by the conventional straw (ST), Cryotop® (MVC) or pullulan film vitrification (PFV) methods were stored in liquid nitrogen for a certain period of time, after which the cryoprotective agents were removed by stepwise dilution. Fresh embryos were used as controls for the non-vitrification group. The vitrified-warmed embryos were incubated in TCM199 with 0.1 mM β-mercaptoethanol and 20% fetal bovine serum for 24 h at 38.5 C in humidified air with 5% CO_2 to evaluate their viability. The survival rate of embryos in the ST group (48.3%) was significantly lower than that of those in the MVC (70.7%), PFV (79.0%) and non-vitrification (94.4%) groups. The oxygen consumption rate after vitrification was significantly lower than that before vitrification in the ST group, but was not significantly different in the MVC and PFV groups. Both the oxygen consumption rates of embryos after warming and the live cell numbers in the ST group were lower than those in the MVC group, while they did not differ significantly between the PFV and MVC groups. There was a correlation between the oxygen consumption rate and the number of live cells in vitrified embryos after warming. Our results demonstrated that *in vivo*-derived porcine embryos could be vitrified using pullulan films.

Key words: Oxygen consumption rate, Porcine embryo, Pullulan film, Vitrification

(J. Reprod. Dev. 56: 279–284, 2010)

Preservation of porcine embryos is important for increasing the effective use of high-quality genetic resources, preventing disease transmission via animals and allowing low-cost transportation of pigs. Due to the extremely high sensitivity of porcine embryos to low temperature, it has been difficult with slow freezing methods to achieve conception rates and litter sizes equivalent to those achieved with artificial insemination [1, 2]. However, it has recently become possible to produce piglets from cryopreserved embryos by using ultra-rapid vitrification methods, such as the minimum volume cooling (MVC) [3, 4], open pulled straw [5], microdroplet [6] and metal mesh vitrification [7] methods.

Pullulan, which is a neutral polysaccharide polymer also known as α-1,4-; α-1,6-glucan, is made from starch and consists of maltotriose units linked in an orderly manner. It has been reported that murine morulae placed on a pullulan film could be vitrified [8]. In general, stepwise dilution is required after warming of vitrified embryos, since vitrification requires a high concentration of cryoprotective agents (CPAs), which makes it difficult, due to their toxicity, to warm the embryos in a straw for direct transfer to recipients. As the pullulan film is soluble in warm water, the vitrification solution can be diluted in a straw, and then the embryos can be directly transferred into a recipient using a straw-attached intrauterine injector.

Several studies on non-surgical embryo transfer in pigs have been reported [9–13], and we have also been working on the development of a non-surgical transfer technique that uses a straw-attached intrauterine injector [14]. Direct transfer via a straw containing vitrified embryos with high viability may be applied to piglet production by non-surgical embryo transfer in an ordinary commercial farm.

Measuring the oxygen consumption rate using a scanning electrochemical microscope may be effective as a non-invasive evaluation of embryo quality [15]. We have previously reported that bovine embryos having a high oxygen consumption rate showed a high conception rate after embryo transfer [16]. In an attempt at embryo cryopreservation using the slow-freezing method, the oxygen consumption rate in embryos significantly decreased after freezing and thawing, indicating that the embryos were damaged by the cryopreservation procedure [16]. However, there are no reports available concerning the oxygen consumption rate before and after cryopreservation with *in vivo*-derived porcine embryos.

In the present study, to investigate the potential for application of the pullulan film vitrification (PFV) method in a non-surgical transfer technique for porcine embryos, we compared the survival rate of porcine embryos preserved by the PFV method with those attained by conventional vitrification methods. We also measured

Received: June 11, 2009
Accepted: November 18, 2009
Published online in J-STAGE: December 25, 2009
©2010 by the Society for Reproduction and Development
Correspondence: N Sakagami (e-mail: sakagami.semi@pref.kanagawa.jp)

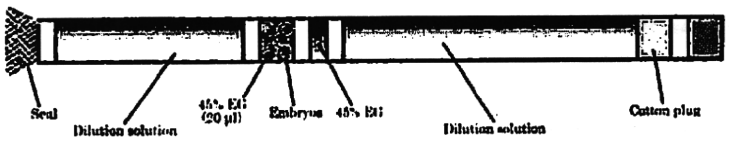


Fig. 1. A straw containing four liquid layers: (from the cotton-plug side) a dilution solution (PB1 + 1.7 M galactose + 0.5% BSA), a 45% EG vitrification solution (PB1 + 45% EG + 7% PVP + 0.5% BSA) that prevents reduction in the concentration of the vitrification solution due to mixing with the diluents of the first layers, a 45% EG vitrification solution containing embryos and a dilution solution. The straw was sealed after being filled with the last dilution layer.

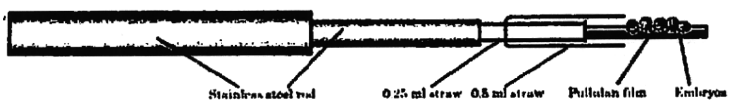


Fig. 2. A stainless steel rod for vitrification equipped with a pullulan film. A stainless steel rod was attached to a short 0.25-ml straw, and a pullulan film was attached to the tip of the straw. The 0.25-ml straw and pullulan film were covered with a case made from a 0.5-ml straw. Embryos and vitrification solution were placed on the pullulan film, and after the embryos were vitrified in liquid nitrogen, the case was slid across to cover the film.

the oxygen consumption rate of embryos before and after vitrification to examine how the rate was influenced by these vitrification methods.

Materials and Methods

Embryo collection from gilts

All animal-related procedures followed in this study were done with the approval of the Institutional Animal Experiment Committee of Kanagawa Prefectural Agriculture Facilities. A total of 39 prepubertal gilts (Landrace and Large White, 5.5–7.3 months old) were used for collection of embryos, as previously described with some modifications [13]. Briefly, superovulation was induced by intramuscular injection of 1500 IU eCG (Peamex, Sankyo, Tokyo, Japan), followed 72 h later by 500 IU hCG (Puberogen, Sankyo). The gilts were artificially inseminated twice, in the afternoon one day after hCG injection and in the morning two days after hCG injection.

In the morning on Day 6 (Day 0=the day of the first artificial insemination), the embryos were recovered from the uterine horns by laparotomy under general anesthesia (4–5% [v/v] halothane) by flushing with PB1 [17] with 0.5% (w/v) bovine serum albumin (BSA; Fraction V, Sigma Chemical, St. Louis, MO, USA) or TALP-Hepes [18]. The recovered embryos were morphologically evaluated under an inverted microscope (× 100), and only blastocysts were used for the experiments. The blastocysts were preserved in PZM-5 [10] supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA) at 38.5 C in humidified air with 5% CO_2 until vitrification.

Vitrification and warming of embryos

The embryos were vitrified using the three different methods and warmed 20–150 days later.

In Method 1, conventional vitrification using a mini-straw (ST) was performed using 0.25-ml crystal straws (IMV Technologies, L'Aigle, France) as described previously [19] with some modifications. Briefly, the embryos were placed in a drop (80 μl) of equilibration solution consisting of PB1 medium with 11% (v/v) ethylene glycol (EG) and 0.5% BSA (11% EG solution) for 5 min at room temperature (20–25 C). After equilibration in 11% EG solution, the embryos were transferred to a vitrification solution consisting of PB1 medium supplemented with 45% EG, 7% (w/v) polyvinylpyrrolidone (MW 10,000, Sigma) and 0.5% BSA (45% EG vitrification solution) preloaded into a straw. Within 40 sec of the initial exposure of the embryos to the 45% EG vitrification solution, the straw was loaded with the solutions, as shown in Fig. 1, heat sealed and placed on a styrene foam board (5 mm thick) floating on liquid nitrogen (LN_2), and the cotton-plug part of the straw was dipped into LN_2 for a quick moment to create ice seeds in the diluent. The straw was then placed on the styrene foam board again for 3 min. After confirming that the 45% EG vitrification solution was transparent and that ice crystals had not been formed, the straw was plunged into LN_2.

After storage in LN_2 for a certain period of time, the straw was warmed in air (25 C) for 5 sec and then in a 40 C water bath for 6 sec. Immediately after warming, the straw was shaken to mix the solutions inside (i.e., first-step dilution). Two minutes after the first-step dilution, the content of the straw was expelled into a dish, and the embryos were subsequently washed by five serial transfer steps of the warmed embryos in PB1 medium supplemented with 0.5% BSA and 6%, 3%, 1.4%, 0.7% or 0% EG for 2 min each.

In Method 2, the MVC method was conducted using a Cryotop® and commercial vitrification and warming solutions (VT101 and VT102, Kitazato BioPharma, Shizuoka, Japan) by the method previously reported [3, 4]. The embryos were contracted by dehydration in an equilibration solution (ES of VT101, Kitazato BioPharma) and kept at room temperature (25 C) for 5 to 10 min until they regained their volume to some extent. The equilibrated

embryos were transferred to a vitrification solution (VS of VT101, Kitazato BioPharma) along with a minimal amount of the equilibration solution. The embryos contracted in the solution were loaded onto the tip of a Cryotop® and directly plunged into LN_2. The length of time from exposure of the embryos to the vitrification solution until storage in LN_2 was less than 60 sec.

After storage in LN_2, the embryos were warmed by immersing the Cryotop® tip into a warming solution (TS of VT102, Kitazato BioPharma) at 38.5 C. After one min, the embryos were transferred to a diluent (DS of VT102, Kitazato BioPharma) at 38.5 C for 3 min and then washed twice in a washing solution (WS of VT102, Kitazato BioPharma) for 5 min each time.

In Method 3, the PFV method was conducted using a pullulan film (1.5 mm wide, 15 mm long and 20 μm thick; Hayashibara Shoji, Okayama, Japan) attached to a stainless steel rod (Fig. 2). The embryos were kept in D-PBS with 20% FBS for 2 min, equilibrated for 4 min in D-PBS with 20% FBS supplemented with 7.5% EG, 7.5% DMSO and 0.3 M sucrose and then transferred to a vitrification solution (EDS30; D-PBS with 15% EG, 15% DMSO, 0.6 M sucrose and 20% FBS). Within 60 sec after transfer to the vitrification solution, the embryos were loaded onto the pullulan film and plunged into LN_2.

After storage in LN_2, the embryos were warmed by submerging the film successively into D-PBS supplemented with 20% FBS and 0.6 M sucrose for 2 min, D-PBS supplemented with 20% FBS and 0.3 M sucrose for 2 min and finally D-PBS with 20% FBS for 2 min.

Viability of embryos

Following warming, the embryos were cultured in TCM199 supplemented with 0.1 mM β-mercaptoethanol and 20% FBS for 24 h at 38.5 C in humidified air with 5% CO_2. The embryos forming a blastocoel were considered viable. As the non-vitrification control, embryos just recovered were also cultured for 24 h in the same medium.

Measurement of the oxygen consumption rate

The oxygen consumption rate using an embryo respirometer (HV-403, Research Institute for the Functional Peptides, Yamagata, Japan), a device developed based on a scanning electrochemical microscope, was measured for some of the embryos 30 min before vitrification and after warming by the method of Abe *et al.* [15]. Briefly, an embryo was placed into the solution for the measurement (ERAM-2, Research Institute for the Functional Peptides) on the flat bottom of a cone-shaped microwell on the plate (RAP-1, Research Institute for the Functional Peptides). A microelectrode (platinum microelectrode RAE-1, Research Institute for the Functional Peptides) was moved close to the embryo, and a voltage of –0.6 V was applied to reduce the oxygen concentration in the solution surrounding the embryo and measure the current generated as a result. The oxygen concentration gradients in the solution surrounding the embryo were measured by scanning the z-axis (i.e., horizontal direction) at a speed of 31.0 μm/sec. The average of two measurements (anterior and posterior sides of the embryo) was considered to be the oxygen consumption rate of the embryo.

Table 1. *In vitro* development of porcine embryos cryopreserved by different vitrification methods

Vitrification method	No. of embryos	
	Cultured	Survived (%)
ST	89	43 (48.3)[a]
MVC	82	58 (70.7)[b]
PFV	81	64 (79.0)[b]
Non-vitrification	54	51 (94.4)[c]

[a–c] Values with different superscripts within each column are significantly different (P<0.05).

Classification of viable and dead cells in embryos

The method of Saha and Suzuki [20] was used to compare the cell viability of the vitrified embryos. After washing with TCM199 containing 20% FBS, the embryos subjected to oxygen consumption rate measurement were warmed to 38.5 C and cultured in a staining solution for 30 min for double staining of viable and dead cells. TCM199 supplemented with 10 μg/ml bisbenzimide (Hoechst 33342, Calbiochem, San Diego, CA, USA) and 10 μg/ml propidium iodide (PI; Sigma) was used as the staining solution. The stained embryos were washed with TCM199 containing 20% FBS, placed on a slide with a small amount of TCM199 with 20% FBS and covered with a cover glass. The embryos were observed through a U-MWU filter (excitation wavelength of 365 nm, barrier filter of 400 nm) under an inverted microscope equipped with a fluorescent light source, and embryos with blue nuclei were counted as viable; those with pink nuclei were counted as dead.

Statistical analysis

The experiment was repeated three or four times for each group. A chi-square test and Fisher's exact probability test was used to compare the viability of embryos. The numbers of cells were subjected to logarithmic transformation before statistical analysis. An analysis of variance (ANOVA) was performed using the General Linear Model (GLM) procedure of SPSS (SPSS 11.5J, User's Guide, SPSS, Tokyo, Japan) followed by the Scheffe test. Linear relationships and correlation coefficients between oxygen consumption rate and the number of live cells in vitrified-warmed embryos were determined by simple regression analysis and Pearson correlation coefficient analysis, respectively. A P value of less than 0.05 was considered statistically significant.

Results

Effect of vitrification procedures on morphological changes in vitrified embryos after warming

The viabilities of embryos after vitrification and warming by the different methods are shown in Table 1. The percentage of embryos that survived in the ST group was significantly lower (P<0.05) than those in the other two vitrification groups and in the non-vitrification group. There was no significant difference in the rates between the MVC and PFV groups. Moreover, the survival rate in the non-vitrification group was significantly higher (P<0.05) than those in the other groups.

Table 2. Oxygen consumption rates of porcine embryos before and after vitrification

Vitrification method	No. of embryos examined	Oxygen consumption rate ($F \times 10^{14}$ /mol s^{-1})	
		Before vitrification	After warming*
ST	10	1.29 ± 0.17^{A}	$0.82 \pm 0.09^{a,B}$
MVC	15	1.15 ± 0.08	1.22 ± 0.08^{b}
PFV	11	0.99 ± 0.08	1.02 ± 0.09^{ab}
Non-vitrification	10	1.32 ± 0.14	-

Mean ± SEM. * Values were measured 30 min after warming. ^{a,b} Values with different superscripts within each column are significantly different ($P<0.05$). ^{A,B} Values with different superscripts within each row are significantly different ($P<0.05$).

Table 3. Cell viability of porcine embryos after vitrification

Vitrification method	No. of embryos examined	Mean cell no. in blastocyst		Ratio of live /total (%)
		Total	Live	
ST	10	70.0 ± 2.5	64.4 ± 3.2^{a}	91.9 ± 2.7^{a}
MVC	13	86.2 ± 4.3	85.9 ± 4.4^{b}	99.5 ± 0.3^{b}
PFV	9	82.3 ± 3.5	80.2 ± 4.3^{ab}	97.0 ± 1.8^{ab}
Non-vitrification	10	70.3 ± 3.7	70.3 ± 3.7^{ab}	100 ± 0^{b}

Mean ± SEM. ^{a,b} Values with different superscripts within each column are significantly different ($P<0.05$).

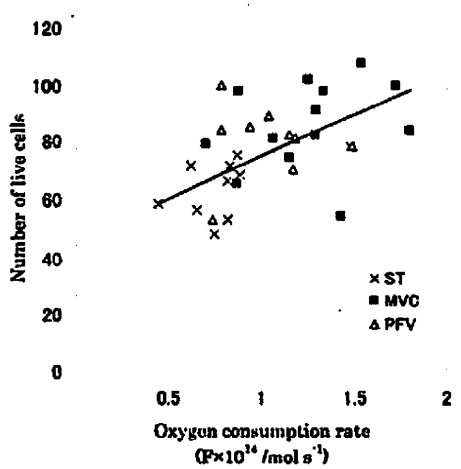


Fig. 3. Correlation between the oxygen consumption rates of vitrified-warmed embryos and number of live cells. There is a significant positive correlation between the oxygen consumption rate and the number of live cells ($P<0.01$ $r=0.496$). Regression lines are drawn to show the relationship between the oxygen consumption rates and numbers of live cells.

Effect of vitrification procedures on the oxygen consumption rates of embryos

The oxygen consumption rate of the vitrified embryos after warming was significantly higher in the MVC group ($P<0.05$) than in the ST group, but in the PFV group, it did not differ significantly from the rates in the other groups (Table 2). The oxygen consumption rate after warming was significantly lower ($P<0.05$) than that at pre-vitrification in the ST group, while there was no significant difference between these rates in the MVC and PFV groups.

Effect of vitrification procedures on cell viability in embryos

Cell viability after vitrification by the different methods is shown in Table 3. The ratio of live to total cell number was significantly lower ($P<0.05$) in the ST groups than in the MVC and non-vitrification groups. There was a positive correlation between the oxygen consumption rate and the number of live cells ($P<0.01$, $r = 0.496$) in all vitrified-warmed embryos (Fig. 3).

Discussion

The present study demonstrated that porcine blastocysts can be vitrified using a pullulan film and that viability, oxygen consumption rate and cell viability were not different from those of embryos vitrified by the MVC method using a Cryotop®.

Since vitrification uses CPAs at high concentrations to prevent intracellular ice crystal formation, their toxicity causes damage to embryos when the vitrification procedure takes a long time. To avoid this problem, several vitrification methods, such as the open-pulled straw [5], microdroplet [6] and metal mesh vitrification [7] methods, have been reported to achieve high viability by making porcine embryos very rapidly pass a critical temperature range at which embryos are injured. The viability of embryos is increased by these methods, as they have the advantages of minimizing the required amounts of CPAs by using special devises and lowering the concentration of CPAs by application of rapid cooling. The present study compared the viability of embryos in the PFV method using pullulan film with that in the MVC method, one of the ultra-rapid vitrification methods, and demonstrated that the viability was 70% or more in the PFV method, which is similar to that in the MVC method. On the other hand, the survival rate of embryos in

the ST group was significantly lower than that in the other groups. The volume of vitrification solution was smaller in the MVC and PFV groups (0.1 μl) than in the ST group (20 μl). Moreover, whereas embryos were placed into LN_2 after exposing them to LN_2 vapor in the ST group, they were directly plunged into LN_2 in the MVC and PFV groups. Thus, the difference in survival may be due to a more rapid temperature decrease in the MVC and PFV groups than in the ST group.

The vitrification procedure for embryo storage does not require a programmable freezer. However, it requires special devises and strict control of temperature and time before plunging the embryos into LN_2 because high concentrations of CPAs are used. Therefore, the number of embryos that can be vitrified in one operation has been limited. Ushijima *et al.* [4] reported that the MVC method allows simultaneous vitrification of 8 to 12 embryos. In pigs, it is desirable to cryopreserve larger numbers of embryos together because 15 to 20 embryos should be transferred into a recipient at one time. In the present study, 5 to 8 embryos could be simultaneously vitrified by using a sheet of pullulan film, and the vitrified embryos on 3 or 4 sheets of the film could be loaded together into a straw. We suggest that the PFV method to cryopreserve porcine embryos is a valuable tool for non-surgical embryo transfer in pigs, since the vitrified embryos can be directly transferred into a recipient. We have obtained normal calves after transfer of bovine blastocysts vitrified using pullulan films and warmed by one-step dilution in straws (our unpublished data). The PFV method appeared to have no adverse effect on porcine embryos in this study. If porcine embryos vitrified using pullulan films can be conveniently diluted and warmed in a straw while maintaining their viability, they may be applied to piglet production by non-surgical embryo transfer under on-farm conditions. Further technical improvements are required to enable one-step dilution in a straw.

In the present study, PFV was applied as an open method in which the solution containing embryos directly contacts LN_2. Storage of embryos in open containers in LN_2 may represent an increased potential for microbial contamination during long-term banking and the risk of disease transmission by embryo transfer [21]. To avoid contamination of pathogens, embryos collected from the same donor should be separately vitrified by the PFV method in its own sterile LN_2 and stored in its own closed containers. An in-straw dilution method after PFV may also enable decrease of the risk of contamination because storage and dilution of vitrified embryos is performed in a sealed straw.

The oxygen consumption rate of embryos vitrified by the three different methods was measured in order to assess the quality of embryos and their potential use in a non-invasive transfer method [16, 22, 23]. To our knowledge, the present study is the first to measure the oxygen consumption rate before and after vitrification of porcine embryos. The oxygen consumption rate after warming was significantly lower than the pre-vitrification levels in the ST group, while there was no significant difference between these rates in the MVC and PFV groups, thus reflecting their respective viabilities. These results were consistent with the viabilities of the embryos after vitrification and warming in these groups, supporting the validity of using the oxygen consumption rate as an index of embryo quality after vitrification and warming.

Dobrinsky *et al.* [24] have reported that success of vitrification depends on the influence of CPAs on morphological and molecular properties of cellular organelles and membranes. In the present study, cell damage of embryos was assessed by a double staining method with Hoechst 33342 and PI, which can distinguish between blue-stained live cells and pink-stained dead cells by the difference in permeability for PI, depending on damage of the cell membrane. The number of live cells in the ST group decreased significantly compared with the MVC group ($P<0.05$), and the ratio of live to total cells was reduced by 5 to 7% in the ST group compared with the other groups. This result may reflect that the survival rate of embryos after warming was markedly lower in the ST group (48.3%) than in the MVC (70.7%), PFV (79.0%) and non-vitrified groups (94.4%). Moreover, both the oxygen consumption rates of embryos just after warming and live cell numbers in the MVC group were higher than those in the ST group. In addition, there was a correlation between the oxygen consumption rate and the number of live cells in vitrified embryos after warming. Trimarchi *et al.* [22] have demonstrated that maturation of mitochondria correlates with an increase in metabolic activity, as reflected in the oxygen consumption rate, and that the oxygen consumption rate reflects the number of cells measured and their mitochondrial activity. Thus, we assumed that the number of live cells could be estimated by the oxygen consumption rate.

In the present study, no difference was found in viability and oxygen consumption rate after warming between the porcine embryos vitrified by the method using pullulan film and those obtained by the previously reported MVC method, indicating that porcine embryos can be vitrified using pullulan film. Non-surgical direct transfer of ultra-rapidly vitrified porcine embryos without using a microscope would be a useful transfer method applicable by individual farmers in the field. Therefore, further studies are desirable on the method for in-straw dilution of embryos.

Acknowledgments

The authors would like to thank Drs H Hoshi and K Misumi for critical suggestions and T Wada, Y Shimizu, H Ozawa, T Kashiwagi, K Tani and H Ayabe for technical assisitance. This study was supported by grants for "Research for the Utilization and Industrialization of Agricultural Biotechnology" (1605) from the Ministry of Agriculture, Forestry and Fisheries of Japan and the "Research and Development Program for New Bio-Industry Initiatives" from the Bio-oriented Technology Research Advancement Institution (BRAIN).

References

1. Polge C, Wilmut I, Rowson LEA. The low temperature preservation of cow, sheep and pig embryos. *Cryobiology* 1974; 11: 560.
2. Pollard JW, Leibo SP. Chilling sensitivity of mammalian embryos. *Theriogenology* 1994; 41: 101–106.
3. Esaki R, Ueda H, Kurome M, Hirakawa K, Tomii R, Yoshioka H, Ushijima H, Kuwayama M, Nagashima H. Cryopreservation of porcine embryos derived from *in vitro*-matured oocytes. *Biol Reprod* 2004; 71: 432–437.
4. Ushijima H, Yoshioka H, Esaki R, Takahashi K, Kuwayama M, Nakane T, Nagashima H. Improved survival of vitrified *in vivo*-derived porcine embryos. *J Reprod Dev* 2004; 50: 481–486.

5. Cuello C, Gil MA, Parrilla I, Tomel J, Vázquez JM, Roca J, Berthelot F, Martinat-Botté F, Martínez EA. *In vitro* development following one-step dilution of OPS-vitrified porcine blastocysts. *Theriogenology* 2004 ; 62: 1144–1152.
6. Misumi K, Suzuki M, Sato S, Saito N. Successful production of piglets derived from vitrified morulae and early blastocysts using a microdroplet method. *Theriogenology* 2003; 60: 253–260.
7. Fujino Y, Kojima T, Nakamura Y, Kobayashi H, Kikuchi K, Funahashi H. Metal mesh vitrification (MMV) method for cryopreservation of porcine embryos. *Theriogenology* 2008; 70: 809–817.
8. Takagi Y, Shimizu M, Kato T, Danguri A, Sakamoto M. Vitrification of mouse morulae by a new method: pullulan film-straw vitrification. *Reprod Fertil Dev* 2004; 16: 183 (abstract).
9. Yonemura I, Fujino Y, Irie S, Miura Y. Transcervical transfer of porcine embryos under practical conditions. *J Reprod Dev* 1996; 42: 89–94.
10. Suzuki C, Iwamura S, Yoshioka K. Birth of piglets through the non-surgical transfer of blastocysts produced *in vitro*. *J Reprod Dev* 2004; 50: 487–491.
11. Martínez EA, Caamaño JN, Gil MA, Rieke A, McCauley TC, Cantley TC, Vazquez JM, Roca J, Vazquez JL, Didion BA, Murphy CN, Prather RS, Day BN. Successful nonsurgical deep uterine embryo transfer in pigs. *Theriogenology* 2004; 61: 137–146.
12. Cuello C, Berthelot F, Martinat-Botté F, Venturi E, Guillouet P, Vázquez JM, Roca J, Martínez EA. Piglets born after non-surgical deep intrauterine transfer of vitrified blastocysts in gilts. *Anim Reprod Sci* 2005; 85: 275–286.
13. Nakazawa Y, Misawa H, Fujino Y, Tajima S, Misumi K, Ueda J, Nakamura Y, Shibata T, Hirayama Y, Kikuchi K. Effect of volume of non-surgical embryo transfer medium on ability of porcine embryos to survive to term. *J Reprod Dev* 2008; 54: 30–34.
14. Fujino Y, Nakamura Y, Kobayashi H, Nakano S, Suzuki C, Yoshioka K. Viability of porcine embryos vitrified by the metal mesh vitrification method after surgical or non-surgical transfer. *Reprod Fertil Dev* 2007; 19: 221–222 (abstract).
15. Abe H, Shiku H, Aoyagi S, Hoshi H. *In vitro* culture and evaluation of embryos for production of high quality bovine embryos. *J Mamm Ova Res* 2004; 21: 22–30.
16. Sakagami N, Akiyama K, Nakazawa Y. The relationship between oxygen consumption rate and pregnancy rate of bovine embryos. *Reprod Fertil Dev* 2007; 19: 225 (abstract).
17. Quinn P, Barros C, Whittingham DG. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J Reprod Fertil* 1982; 66: 161–168.
18. Bavister BD, Leibfried ML, Lieberman G. Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biol Reprod* 1983; 28: 235–247.
19. Kobayashi S, Takei M, Kano M, Tomita M, Leibo SP. Piglets produced by transfer of vitrified porcine embryos after stepwise dilution of cryoprotectants. *Cryobiology* 1998; 36: 20–31.
20. Saha S, Suzuki T. Vitrification of *in vitro* produced bovine embryos at different ages using one- and three-step addition of cryoprotective additives. *Reprod Fertil Dev* 1997; 9: 741–746.
21. Bielanski A, Bergeron H, Lau PCK, Devenish J. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. *Cryobiology* 2003; 46: 146–152.
22. Trimarchi JR, Liu L, Porterfield DM, Smith PJS, Keefe DL. Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. *Biol Reprod* 2000; 62: 1866–1874.
23. Lopes AS, Madsen SE, Ramsing NB, Lovendahl P, Greve T, Callesen H. Investigation of respiration of individual bovine embryos produced *in vivo* and *in vitro* and correlation with viability following transfer. *Hum Reprod* 2007; 22: 558–566.
24. Dobrinsky JR. Cellular approach to cryopreservation of embryos. *Theriogenology* 1996; 45: 17–26.

Journal of the American Association for Laboratory Animal Science
Copyright 2010
by the American Association for Laboratory Animal Science

Vol 49, No 2
March 2010
Pages 160–166

Lactate and Adenosine Triphosphate in the Extender Enhance the Cryosurvival of Rat Epididymal Sperm

Hideaki Yamashiro,[1,3,*] Masaaki Toyomizu,[2] Motoi Kikusato,[2] Natsuki Toyama,[1] Satoshi Sugimura,[1] Yumi Hoshino,[1] Hiroyuki Abe,[4] Stefan Moisyadi,[3] and Eimei Sato[1]

We evaluated the cryosurvival of rat epididymal sperm preserved in raffinose–modified Krebs-Ringer bicarbonate–egg yolk extender supplemented with various energy-yielding substrates (glucose, pyruvate, lactate, and ATP) and assessed the effect on sperm oxygen consumption. The incubation of sperm at 37 °C for 10 min in lactate-free extender decreased sperm motility and oxygen consumption before and after thawing compared with those of sperm in glucose- and pyruvate-free mediums. We then focused on the effect of supplementing the extender with lactate (0, 10.79, 21.58, 32.37, and 43.16 mM) and found that sperm frozen and thawed in extender supplemented with 32.37 mM lactate exhibited the highest motility. When we supplemented extender containing 32.37 mM lactate with ATP (0, 0.92, 1.85, 3.70, and 5.55 mM), sperm frozen and thawed in the extender supplemented with 1.85 mM ATP exhibited considerably higher motility and viability than those of sperm frozen and thawed in ATP-free extender. These results provide the first evidence that supplementation of the raffinose-modified Krebs–Ringer bicarbonate–egg yolk extender with 32.37 mM lactate and 1.85 mM ATP increases of number of motile sperm before freezing and enhances the cryosurvival of rat sperm. These supplements to the extender may enhance sperm cryosurvival by improving the metabolic capacity of sperm before freezing.

Abbreviations: mKRB, modified Krebs–Ringer bicarbonate.

We previously showed that freezing rat epididymal sperm in an extender of raffinose dissolved in modified Krebs–Ringer bicarbonate (mKRB) solution containing egg yolk enhances their cryosurvival of sperm as measured by viability and acrosomal integrity; this finding suggested that a mKRB-based freezing extender containing glucose, pyruvate, and lactate can protect sperm against freezing injury.[33] A possible reason for this finding is that sperm in this extender retain high metabolic capacity before freezing which, in turn, may enhance the cryosurvival of rat sperm. However, the mechanism by which the mKRB-based extender promotes the metabolic capacity and cryosurvival of rat sperm is unclear.

The process of cryopreservation imposes numerous stresses on not only the physical features of sperm but also the energy production to support motility before and after freezing, and improving energy production in frozen–thawed sperm is important for successful cryopreservation.[19] Sugars play various roles in sperm extender solution, including providing an energy substrate for sperm during cooling and acting as a cryoprotectant.[1] The beneficial effects of adding glucose to the extender on the viability of frozen–thawed sperm have been reported for various species;[2] this nutritional effect may involve the synthesis and provision of ATP through the glycolytic pathway to provide the energy required for sperm motility. Therefore, glucose may play a key role in generating energy in motile sperm and preventing freezing damage.

The addition of an exogenous substrate (such as lactate) improved the sperm motility characteristics of cattle,[11] boars,[14,21] and rabbits.[29] A previous study[9] showed that a shuttle involving the redox couple lactate–pyruvate and lactate dehydrogenase isozyme C_4 is active in rat and rabbit mitochondria but not in mouse mitochondria. This finding suggests that sperm from various species, including rats, may find lactate a suitable substrate for maintaining the energy production and consumption as well as oxygen consumption. However, which of the major biochemical pathways—glycolysis or oxidative phosphorylation—is involved in supplying energy to mobilize sperm has been a long-lasting debate, because the pathway of energy production is species-specific.[8,28,32] Regardless of the identity of the pathway involved in energy supply, the development of an appropriate freezing extender likely would improve sperm motility and survival during cryopreservation through enhancement of the metabolic capacities of sperm. However, no previous studies have reported on the optimal components, especially the energy substrates, of a cryodiluent for the freezing of rat sperm.

Sperm may attain access to eggs by mobilizing metabolic energy production in the form of ATP to drive motility.[30] ATP is hydrolyzed by the dynein adenosine triphosphatase, which converts the chemical energy of ATP into mechanical energy used for the movement of sperm.[3,15] ATP can have several downstream effects leading to improvement in the motility of sperm by means of an increase in the calcium level.[10,17,18,20,25,27] Moreover, the amount of ATP required for metabolic energy is higher in the cytosol than the mitochondria.[16] Therefore, sup-

Received: 02 Jul 2009. Revision requested: 06 Aug 2009. Accepted: 24 Aug 2009.
[1]Laboratory of Animal Reproduction and [2]Laboratory of Animal Nutrition, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan; [3]Institute for Biogenesis Research, Department of Anatomy and Reproductive Biology, John A Burns School of Medicine, University of Hawaii, Honolulu, HI; [4]Graduate School of Science and Engineering, Yamagata University, Yamagata, Japan.
[*]Corresponding author. Email: hideaki66@hawaii.edu

plementation of the freezing extender with exogenous ATP may improve the cryosurvival of rat epididymal sperm.

Here we evaluated the cryosurvival and parameters of mitochondrial activity, including oxygen consumption, of rat sperm diluted in raffinose–mKRB–egg yolk extender supplemented with various energy-yielding substrates, including glucose, pyruvate, lactate, and ATP. In addition, we identified the optimal energy substrates and other components of a cryodiluent for the freezing of rat sperm.

Materials and Methods

Principles of laboratory animal care were followed during this study, and all procedures were conducted in accordance with guidelines of the Ethics Committee for Care and Use of Laboratory Animals for Research of the Graduate School of Agricultural Science (Tohoku University, Japan). Wistar rats were used throughout the experiments. Animals were kept in polycarbonate cages (25 × 40 × 20 cm) under controlled conditions with lights on at 0800 and off at 2000. They were given food and tap water ad libitum.

Preparation of the raffinose–mKRB–egg yolk extender. The basic extender used in this study was the raffinose–mKRB–egg yolk freezing solution defined previously;[33] it comprised 0.1 M raffinose (Sigma, St Louis, MO), 94.6 mM NaCl (Wako Pure Chemical Industries, Osaka, Japan), 4.78 mM KCl (Wako), 1.71 mM $CaCl_2 \cdot 2H_2O$ (Wako), 1.19 mM $MgSO_4 \cdot 7H_2O$ (Wako) 1.19 mM KH_2PO_4 (Wako), 25.07 mM $NaHCO_3$ (Wako), 21.58 mM sodium DL-lactate (Sigma), 0.5 mM sodium pyruvate (Wako), 5.56 mM glucose (Wako), 50 µg/mL streptomycin (Sigma), and 75 µg/mL penicillin (Sigma); egg yolk was separated from the albumin, and 20% (v:v) egg yolk was added to the raffinose–mKRB solution. Egg yolk lipids were solubilized by adding 0.04% (w:v) SDS (Wako) to the solution. The solution was centrifuged twice at 7000 × *g* for 30 min. The pH of the solution was adjusted to 7.3 with HCl and its osmotic pressure to 400 mOsm. The supernatant was aspirated and filtered through a 0.45-µm membrane filter (Sartorius, Goettingen, Germany).

Evaluation of sperm motility parameters. Sperm motility parameters were assessed by using a sperm motility analysis system (version 1.0, Kashimura, Tokyo, Japan) and a 10-µm deep Makler chamber (Sefi Medical Instruments, Haifa, Israel); the protocol was described previously.[6] At least 100 sperm and 5 fields were assessed by the sperm motility analysis system for each treatment group. The following parameters were assessed in this study: motility (%), straight line velocity (µm/s), curvilinear velocity (µm/s), amplitude of lateral head displacement (µm), and beat cross frequency (Hz).

Evaluation of sperm acrosome integrity. The acrosomal integrity of fresh and frozen–thawed sperm was assessed by staining with FITC-conjugated peanut agglutinin (Wako) according to the procedure described previously.[33]

Collection of rat epididymal sperm. Both caudae epididymides were excised from 24 sexually mature male Wistar rats older than 15 wk. The excised epididymis was rinsed and carefully blotted free of blood and adipose tissues. A small part of the caudae epididymides tract was excised with fine scissors. The droplet of sperm that welled up was transferred to a 1.5-mL microfuge tube containing 1 mL of freezing medium at 37 °C. After 5 min, the solution was examined macroscopically to verify that sperm were dispersed adequately.

Cryopreservation and thawing. ***Experiment 1a.*** In this experiment, we investigated the effect of the substrates glucose, pyruvate, and lactate in raffinose–mKRB–egg yolk freezing extender on the motility characteristics of fresh sperm after collection and frozen–thawed sperm. Sperm from both the caudae epididymides from 3 rats were used in this experiment. Immediately after collection, aliquots of sperm were exposed to the following 5 solutions: raffinose–mKRB–egg yolk extender containing the substrates glucose, pyruvate, and lactate (control); glucose-free extender; pyruvate-free extender; lactate-free extender; and substrate-free extender. The osmotic pressure of these solutions was adjusted to 400 mOsm with sucrose (Wako) and the pH to 7.3 with HCl. Each sperm suspension was incubated at 37 °C for 5 min to allow the sperm to disperse, and the sperm concentration and motility parameters then were evaluated by the sperm motility analysis system. The sperm were processed and frozen by using a modification of a previously published protocol.[33] The diluted sperm samples were cooled at 5 °C for 90 min. The sperm samples were further diluted 1:1 with each extender containing 1.5% of a commercial cryoprotectant (Equex STM, Nova Chemical Sales, Scituate, MA) to obtain a sperm concentration of 5×10^6 sperm/mL and then were equilibrated at 5 °C for 30 min before freezing. Afterward, the samples were loaded into standard 0.5-mL straws and the straws were heat-sealed. The straws were placed in liquid nitrogen vapor for 10 min, plunged into liquid nitrogen (–196 °C), and stored for 3 d at this temperature. The straws were thawed rapidly by holding them in water (37 °C) for 10 s. The sperm were transferred to a 1.5-mL microfuge tube and incubated at 37 °C for 5 min, after which motility parameters after thawing were assessed by using the sperm motility analysis system. The acrosome status of frozen–thawed sperm was assessed by staining with FITC-conjugated peanut agglutinin.[1]

Experiment 1b. Building on the results of Experiment 1a, this experiment was conducted to analyze the characteristics of fresh and frozen–thawed sperm in the raffinose–mKRB–egg yolk extender containing various concentrations of lactate. Sperm from both caudae epididymides from 3 rats were used. Aliquots of sperm were suspended in 1 mL raffinose–mKRB–egg yolk extender containing 0, 10.79, 21.58, 32.37, or 43.16 mM lactate; all of these solutions had an osmotic pressure of 400 mOsm and a pH of 7.3, except the solution containing 43.16 mM lactate (430 mOsm and pH 7.3). The procedures followed for cryopreservation and evaluation of sperm were the same as described for experiment 1a.

Experiment 1c. This experiment was designed to compare the cryosurvival of rat sperm frozen in raffinose–mKRB–egg yolk extender solution containing 32.37 mM lactate supplemented with various concentrations of ATP. Both caudae epididymides from 3 male rats were used in this experiment. After collection, sperm was divided into 5 aliquots and suspended in 1 mL of extender solution containing 32.37 mM lactate and 0, 0.92, 1.85, 3.70, or 5.55 mM ATP (400 mOsm and pH 7.3). The freezing protocol and evaluation of sperm were the same as described previously. For the evaluation of sperm viability, frozen–thawed sperm samples were incubated in a water bath at 37 °C for 5 min. For each treatment, 3 samples were evaluated after 1, 2, and 3 h of incubation to determine sperm motility, straight-line velocity, curvilinear velocity, and amplitude of lateral head displacement.

Measurement of oxygen consumption of sperm. ***Experiment 2a.*** The aim of this experiment was to assess the effect of substrates in the raffinose–mKRB–egg yolk medium on the mitochondrial activity of sperm. Sperm was collected from 5 mature male rats and extended in substrate-free raffinose–mKRB–egg yolk medium at 37 °C. The sample was incubated for 5 min to allow the sperm to disperse and then equal volumes were resuspended in each of the following solutions: raffinose–