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Comparison between Isolate® gradient and Swim-up Procedures for Sperm Preparation: Effects on Freeze-thawing in Normal Semen Sample

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정상 정자에서 Isolate® gradient와 Swim-up 방법의 비교연구: 동결 및 융해시 미치는 영향

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연구목적: Isolate® gradient와 swim-up 방법이 정자의 형상 및 정밀정자형태 (strict morphology)에 미치는 영향을 비교분석하고, 이러한 정자처리방법이 정자의 동결-융해과정에 미치는 영향을 비교하고자 하였다.

연구재료 및 방법: 20명의 정상 정자를 대상으로 하였으며 각각의 정자는 두가지 정자처리방법으로 나누어 정자의 형상과 정밀정자형태를 컴퓨터를 이용한 정자자동분석기를 통하여 측정하였고, 동결보호제로는 TYB 용액을 사용하였으며, 동결 및 융해는 cryo Magic 사의 기계를 사용하였다. 통계는 SPSS PC+ (version 7.0)를 이용하였으며 통계학적인 유의성은 $p<0.05$로 하였다.

결 과: 정자의 농도는 Isolate® gradient 처리군이 swim-up 처리군보다 유의성 있게 높았으나 (51.2±40.1, 156.6±64.3), 운동성 VCL, VSL, VAP, Linearity, 및 ALH는 swim-up 처리군에서 유의성 있게 높았다. 정밀정자형태는 swim-up 처리군과 Isolate® gradient 처리군에서 차이가 없었다 (53.7±6.8 vs 50.3±9.1%). 동결-융해과정 중 두가지 정자 처리군에서 정자의 형상들은 swim-up 처리군에서 전반적으로 높은 양상을 보였으나, 정밀정자형태는 Isolate® gradient 처리군이 swim-up 처리군 보다 감소율이 컸지만 두군간에 유의한 차이는 없었다 (12.8±8.5 vs 8.6±6.6).

결 론: 정상 정자에서 swim-up 방법이 Isolate® gradient 방법보다 정자 회수율은 우수하였으나, 동결-융해과정 중 정밀정자형태에는 차이가 없어 두 방법을 상호보완적으로 사용할 수 있을 것으로 사료된다.

Key Words: Swim-up, Isolate® gradient, Sperm cryopreservation

Sperm preparation methods are currently available to select motile sperm include swim-up,[1] continuous or discontinuous Percoll gradient[2] and glass wool fiber filtration.[3]

Conventionally, the Percoll technique, which separates spermatozoa according to their density, favours the isolation of the motile and morphological normal spermatozoa. It is likely that Percoll centrifugation separates sperm on the basis of density, selecting sperm with good nuclear morphology that are more dense. The swim-up technique, however, separates the sample into motile and non-

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motile fractions.

Numerous reports provide evidence to support the superiority of these two methods. Some investigators reported that the Percoll gradient was superior to the swim-up.[4-6] However, other investigators found that regarding motility and morphology[7,8] swim-up selected sperm of higer quality than those from the Percoll gradient.

Recently, it has been reported that Percoll is no longer recommended for use in Assisted Reproduction procedures in humans. Because Percoll uses silica particles coated with PVP, it might be slightly toxic and tends to loosen from the silica in salt solution. It can create genetic problems. Therefore, it is necessary to substitute Percoll for other equivalent materials. Thus, in this study we used the Isolate® gradient method instead of Percoll. Isolate®, a density gradient (50%, 90%), is a processsed colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane in a HEPES-buffered HTF. Isolate® gradient may be harmful than Percoll in the preparation of sperm.

Several reports have shown that cryopreservation is detrimental effects on sperm motility and morphology.[9,10] However, there are few reports on the effects of freeze-thawing.

No previous studies have documented a comparison between an Isolate® gradient and swim-up for sperm preparation on sperm parameters, morphology and their effects on the freeze-thawing procedure in normal sperm. This study will establish and compare the sperm preparation methods in normal sperm and abnormal sperm.

MATERIALS AND METHOD

1. Materials

From October 2000 to Jan 2001, samples were taken from twenty normal males who visited IVF clinics. The sperm parameters were anlayzed by SAIS (Sperm Analysis Imaging System; Plus version 10.0; Medical Supply Co). The sperm movement characteristics are described below:

Curvilinear Velocity (VCL) is the total distance travelled divided by the total time the cell was tracked. Straight-Line Velocity (VSL) is the straight line distance between the start and end of the observed track. Average Path Velocity (VAP) is the velocity along the average path of the spermatozoon. The progression ratios, expressed as integer percentages, linearity can be calculated by VSL/VCL x100. Amplitude of Lateral Head Displacement (ALH) is calculated from maximal deviation of the sperm head from the mean trajectory.

2. Methods

1) Swim-up

Semen was mixed gently with an equal volume of Ham's F-10 fortified with 10% SSS (serum substitute supplement, Irvine scientific) and centrifuged at 1000 rpm for 10 minutes.

The pellet was resuspended in the same medium, layered on top with approximately 1 ml of medium, and incubated for 1 hour at 37℃. The upper one-third of the supernatant was then collected.

2) Isolate® gradient method

Using a sterile pipette, 1.5 ml of the lower layer was transferred into a sterile conical tube. Using a new sterile pipette, an equal volume of the upper layer was transferred on top of the lower layer. Liquefied semen (was placed on the upper layer. The layers were centrifuged for 20 minutes at approximately 1800 rpm. The pellet was carefully exposed by aspirating off the upper and) lower layer.

3) Sperm morphology

The aliquots from raw semen, swim-up and Isolate® gradient samples were taken for smears, dried in air. The staining procedure was Diff Quick stain. The sperm were calculated using

SAIS semen analyzer according to Kruger's strict criteria.[11]

In total more than 400 sperm were observed. The sperm was considered normal when normal morphology of head, neck and tail of sperm was more than 14%. That is the head should be smooth and oval with the long axis measuring 5~6 μm and short axis measuring 2.5~3.5 μm; the acrosome should be well defined and constitute 40~70% of the head; midpiece should be slender; the tail should be unifrom, uncoiled, thinner than the midpiece, and approximately 45 μm in length. There should be no cytoplasmic droplets larger than one-half the area of the head. There should be no midpiece or tail defects.

4) Cryopreservation of sperm

A cryoprotectant (TYB) was added to the sperm samples that were treated by the swim-up and Isolate® mentods. The ratio of sample to TYB solution was 1:1. TYB solution was added gently along the wall of the tube and mixed gently. The sperm and TYB solution underwent the cryopreservation procedure.

The cryopreservation procedure was initiated by adjusting the chamber temperature to +20℃. As soon as the last straw was loaded and sealed with powder, the cooling procedure was started. Straws placed in the freezing chamber (+20℃) and cooled at 2℃/min to -7℃, then held for 10 min and cooled at -10℃/min to -80℃ and held for 10 min at -80℃. Then they were transferred to a pre-preserved marked gobblet in an LN_2 tank.

5) Thawing of sperm

The straw was removed from the LN_2 tank and held in air for 30 sec, then plunged into a 37℃ water bath for 40~50 sec. The straw was removed from the water bath and wiped dry and cut seals off ends of the straw. The contents were gently expelled into pre-warmed media (Ham's F10 + 10% SSS) in a tube. The mixture was centrifuged at 1000 rpm for 10 minutes. The sample was analyzed within 30 min after thawing.

3. Statistical analysis

Statistical analysis was carried out using the SPSS PC^+ statistical package at a 5% level of significance. Differences across all parameters were analysed with Student's paired t-test.

RESULTS

Compared to the raw samples, the sperm concentration in the swim-up samples was signifycantly smaller than that in the Isolate® sample. The percentage of progressive motility was greater in the swim-up than in the Isolate® samples. Individual variation in motility was higher in the Isolate® gradient than swim-up after sperm preparation (SD: ±27.9, 7.9; respectively). Of the movement characteristics of sperm including VCL, VSL, VAP, Linearity and ALH, VCL, VAP and ALH were significantly higher in the swim-up samples than in the Isolate® gradient samples. The percentage of strict morphology was significantly increased in the swim-up, but all the strict morphology levels after both procedures fell within the normal range (Table 1).

Comparisons of the two sperm preparation methods after freeze-thawing are summarized in Table 2. After freeze-thawing, the concentration of sperm did not significantly change in either preparation method. The percentage of progressive motility was greater in swim-up than in the Isolate® gradient after freeze-thawing, but the difference before and after the procedure was not significant. VCL, VSL and ALH decreased more significantly in the swim-up procedure after freeze-thawing. The strict morphology in swim-up decreased from 53.7±6.8% before freezing to 44.0±3.4% postthawing and in the Isolate® gradient from 50.3±9.1% to 37.5±5.1%, but the changes of sperm parameters of both preparation methods after the postthawing

Table 1. Comparison of sperm parameters between swim-up and Isolate® procedure (n=20)

	Raw	Swim-up	Isolate®
Conc. (x10^6/ml)	106.7±35.1	51.2±40.1*	156.6±64.3
Motility (%)	59.9±14.7	86.2±7.9*	70.9±27.9
VCL (μm/s)	35.4±9.4	63.4±16.0*	39.3±18.8
VSL (μm/s)	17.1±6.2	36.6±10.3	19.5±11.5
VAP (μm/s)	24.6±6.9	44.4±10.9*	26.6±13.9
Linearity	48.0±7.8	58.4±10.6*	47.1±10.5
ALH (μm)	3.3±0.6	4.6±1.1*	3.5±1.1
SM (%)	43.3±7.1	53.7±6.8*	50.3±9.1

Values are mean ± SEM, *$p<0.05$. compared with raw sperm

Table 2. Effect of the freeze-thawing procedure on the sperm parameters in swim-up and Isolate® gradient procedures (n=20)

	Swim-up	Isolate	Thawing (swim-up)	Thawing (Isolate®)	△swim-up	△Isolate®
Conc. (x10^6/ml)	51.2±40.1	156.6±64.3	30.7±13.9	115.4±47.7	24.5±35.9	48.7±62.3
Motility (%)	86.2±7.9	70.9±27.9	41.5±11.7†	37.2±29.3	44.7±12.8	33.6±19.4
VCL (μm/s)	63.4±16.0	39.3±18.8	34.6±9.8†	29.6±10.8	18.8±14.5	9.7±11.7‡
VSL (μm/s)	36.6±10.3	19.5±11.5	18.1±5.9†	13.4±6.9	18.5±12.0	6.1±7.4‡
VAP (μm/s)	44.4±10.9	26.6±13.9	22.5±7.1†	17.9±8.4	21.8±12.0	8.7±8.7
Linearity	58.4±10.6	47.1±10.5	51.1±9.0	42.6±10.5	7.0±12.0	4.5±7.0
ALH (μm)	4.6±1.1	3.5±1.1	3.1±0.5†	3.0±0.6	1.4±0.7	0.5±0.8‡
SM (%)	53.7±6.8	50.3±9.1	44.0±3.4†	37.5±5.1	8.6±6.6	12.8±8.5

Values are mean ± SEM, †$p<0.05$. compared with two sperm preparation methods
‡$p<0.05$. compared with difference of two sperm preparation methods after thawing

procedure were not significant (8.6±6.6, 12.8± 8.5, respectively) (Table 2).

DISCUSSION

Many groups currently use two methods for the separation of motile spermatozoa, swim-up and Percoll gradient, and the the results has been discarrsed.

Recently, it has been reported that Percoll gradient is no longer recommended for use in Assisted Reproduction procedures in humans. Percoll uses silica particles coated with PVP. It may be slightly toxic and tends to loosen from the silica in salt solution. It can create a genetic problems. Therefore it is necessary to substitute Percoll for other equivalent materials. Thus, in this study we used the Isolate® gradient method instead of Percoll. Isolate®, a density gradient (50%, 90%), is a processed colloidal suspension of silica particles stabilized with covalently bound hydrophilic silane in a HEPES-buffered HTF. Isolate® gradient is less harmful than Percoll in the preparation of sperm. Our study compared swim-up and Isolate® gra-

dient procedures for sperm preparation on the sperm parameters, strict morphology and also their changes after freeze-thawing with the two methods on normal sperm.

The percentage of progressive motility was greater in sample prepared by the swim-up than in sample prepared by the Isolate® procedure in normal sperm. These findings agree with the results of Chen et al (1995)[7] and Englert et al (1992).[8] But Moohan et al (1995)[4] reported that the Percoll gradient method selected spermatozoa with better motion characteristics, more hyperactivation, and improved longevity, compared with swim-up. Van der Zwalmen et al (1991)[5] reported that the sperm selected by Percoll gradient resulted in higher pregnancy rate than swim-up in IVF. However, other investigators did not confirm the benefit of the Percoll gradient in IUI and IVF.[8,11]

The reasons for the varied results are unclear, but Chen et al (1995)[7] suggested it may be related to the variable methods in the Percoll gradient procedure.

Strict morphology (SM) as assessed by strict criteria is a good predictor of oocyte fertilization. This method can be recommended as the method of choice for assisted reproductive technology laboratories.[13,14] Some authors reported Percoll gradient separated more normal forms than the swim-up procedure.[4-6] However, some authors found that the swim-up technique was signifycantly superior for selection of normal forms than the Percoll gradient technique.[8,15] Our results showed that the SM after the swim-up and Isolate® gradient procedure, even though freeze-thawing was performed, did not significantly decrease. These differences can be explained either by the lack of uniformity in the Percoll gradient preparation, or by the classification used to describe morphology.

The VCL, VSL, VAP, and ALH of sperm were significantly greater with the swim-up than with Isolate® gradient procedure, and similar to results reported by Chen et al (1995).[7] After freeze-thawing procedures, the difference of decreased sperm movement characteristics were greater in swim-up, but the value itself was greater in swim-up procedure. These movement characteristics are reported to be a better predictor of the fertility outcome.[16] From this point of view, the swim-up procedure selects sperm with superior quality from normal sperm. But these results do not indicate that Isolate® gradient procedure can be related nuclear DNA damage.

Cyropreservation is known to impair sperm motility and morphology.[9,10] Postthaw sperm survival was about 52%.[17] Our results appear to reflect this. The postthaw sperm survival in swim-up and Isolate® gradient procedure was 48% and 52%, respectively (the data is not shown).

Hammadeh et al (1999)[18] reported that the freeze-thawing procedure significantly affects sperm morphology. However, our study showed that there after postthawing, strict morphology tended to decrease, but the decreased levels were within normal levels. Comparison between the two preparation methods showed no significant changes observed with regard to strict morphology after cryopreservation.

We can draw limited valid conclusions from our study because the number of cases presented here were too small and were not compared with abnormal sperm. Such a study including abnormal sperm is currently under way in our clinic.

In conclusion, in normal sperm, the sperm collected by the swim up procedure was of higher quality regarding motility than that prepared by the Isolate® gradient method. But the changes of sperm parameters after freeze-thawing by two preparation methods was not significantly difference. The strict morphology was also not significantly impaired after cryopreservation.

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