CLINICAL STUDY

Comparative Evaluation of Carbon Dioxide and Carbon Dioxide Free System in Sperm Extraction by Swim-up Technique

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Received 25 October 2006; received in revised form 24 February 2007; accepted 02 March 2007; published online 01 May 2007

Abstract

Objective: To compare the effectiveness of carbon dioxide and carbon dioxide-free incubation system in extracting motile, morphologically normal and chromatin intact sperm populations from the individual semen samples.
Materials and Methods: Semen samples were obtained from normozoospermic men presenting for infertility evaluation. Individual samples were divided into two equal aliquots for sperm wash using CO_2 and CO_2 free system. Sperm motility, viability, morphology and DNA integrity were assessed in the washed spermatozoa. Sperm DNA integrity was evaluated by acridine orange binding and expressed as the percentage of spermatozoa demonstrating denatured DNA.
Results: There was no significant difference in the mean sperm count, motility and morphology of sperms extracted by two systems and both CO_2 and CO_2 free systems significantly eliminated the number of sperm with denatured DNA.
Discussion: The CO_2 free system is comparable to conventional CO_2 incubation system for swim-up technique in recovering spermatozoa with enhanced motility, morphology and higher DNA integrity.

Keywords: sperm wash, water bath, CO_2 incubator, sperm chromatin, IUI

Özet

Swim-up Tekniği ile Sperm Ekstraksiyonunda Karbondioksitli ve Karbondioksitsiz Sistemlerin Karşılaştırmalı Değerlendirilmesi

Amaç: Semen örneklerinden motil, morfolojik olarak normal ve kromatini intakt sperm ekstraksiyonunda karbondioksitli ve karbondioksitsiz inkübasyon sistemlerinin etkinliğini karşılaştırmak.
Materyal ve Metot: İnfertilite değerlendirmesi için başvuran normozoospermik erkeklerden semen örnekleri alındı. Tüm örnekler CO_2'li ve CO_2'siz sistemle yıkanmak üzere eşit olarak ikiye bölündü. Yıkanmış spermatozoada sperm motilitesi, viabilite, morfoloji ve DNA bütünlüğü değerlendirildi. Sperm DNA bütünlüğü turuncu akridin boyası kullanılarak değerlendirildi ve denatüre DNA sergileyen spermatozoa yüzdesi olarak ifade edildi.
Sonuçlar: İki farklı sistemle elde edilen örneklerin ortalama sperm sayısı, motilite ve morfolojisinde anlamlı farklar yoktu ve her iki sistem denatüre DNA'lı sperm sayısını anlamlı ölçüde elimine etti.
Tartışma: Swim-up tekniğinde artmış motilite, morfoloji ve DNA bütünlüğü olan spermatozoa elde etmek için CO_2'siz sistem, geleneksel CO_2'li sistem ile karşılaştırılabilir sonuçlar vermektedir.

Anahtar sözcükler: sperm yıkama, su banyosu, CO_2 inkübatörü, sperm kromatini, IUI

Introduction

Human spermatozoa vary widely in their characteristics from individual to individual and from sample to sample within individuals (1). The seminal plasma contains factors that inhibit capacitation, acrosome reaction and fertilization (2,3), and therefore an ideal sperm preparation method in medically assisted conception should select morphologically normal, motile sperm from the ejaculate and eliminate the inhibitory factors. It should also minimize contamination and iatrogenic damage to sperm during processing. Several techniques have been developed to remove the undesired sperm, round cells, debris, and thereby increase the overall sperm quality (4-8). The conventional wash technique involves centrifugal separation of sperma-

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tozoa followed by incubation at 37°C using 5% carbon dioxide in the incubator at a pH 7.4.

Although, the CO_2 incubators are routinely used in various sperm extraction methods including sperm preparation for intra uterine insemination program, the idea of using water bath for sperm wash is new and there has been no study comparing the sperm yield obtained by water bath and conventional incubation using CO_2 incubator. Hence, in the present study, we optimized a CO_2-free culture system for sperm wash followed by swim up using water bath system. Thereafter, the effectiveness of two different systems i.e. the CO_2 (incubation using CO_2 incubator) and the CO_2-free (incubation with water bath) systems in extracting motile sperm populations from a single semen sample was assessed. In addition, we compared the ability of the CO_2 and the CO_2-free systems for the extraction of morphologically normal and chromatin condensed spermatozoa (spermatozoa with condensed/normal chromatin) from native semen samples to determine their influence on the sperm recovery.

Materials and Methods

Subjects

In this prospective study, semen samples (n=25) were obtained from consecutive normozoospermic men presenting for infertility evaluation at the Division of Reproductive Medicine, Kasturba Medical College from February 2006 to June 2006. Semen samples were produced by masturbation after 3-5 days of ejaculatory abstinence.

Semen analysis

The semen samples were kept in laminar flow at room temperature for liquefaction after which, the standard semen parameters (count, motility and morphology) were evaluated according to World Health Organization (WHO) guidelines (9). Even though the motility will give an idea about viability one can not correlate the effect of any treatment or application of any method for sperm preparation on the viability of spermatozoa since all the non-motile sperms are not dead. For estimation of sperm viability, semen samples were mixed with eosin and nigrosin stain and thin smears were prepared on clean glass slides. The slides were air dried and the vitality was scored under light microscope oil immersion. A total of 200 spermatozoa were counted and the percentage of viability was calculated. The study was approved by the Institutional Ethical Committee and the patient information for this study was kept confidential.

Acridine orange (AO) staining of sperm

Smears were prepared from fresh and washed spermatozoa on a clean grease-free slide and air dried. The slides were fixed in Carnoy's fixative (1:3 glacial acetic acid and absolute methanol) for 2 h. Slides were then stained with AO (0.1% AO in 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M disodium orthophosphate) at pH 2.5 for 5 min in the dark as described by Royere et al. (10) with minor modifications (11). Excess stain was removed by washing in citrate buffer and observed under fluorescent microscope. At least 500 spermatozoa were counted from each slide and the percentage of normal and abnormal chromatin condensation was calculated.

Sperm extraction

Two ml of semen sample from each patient was used for sperm extraction. The samples were divided into two aliquotes (1ml each) and one part was washed using EBSS medium containing bicarbonate buffer supplemented with 0.1% human serum albumin followed by incubation at 37°C in CO_2 incubator (Heracell 150, Germany) for one hour. The other part of the semen was washed in the EBSS medium containing bicarbonate buffer supplemented with 0.1% HSA and 15 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and then incubated for one hour in the water bath (HAAKE, Germany) for sperm migration by swim up. For both systems, the sperm pellet was overlaid using 500 µl of the respective medium. The spermatozoa were collected after one hour without disturbing the pellet. The sperms were assessed for their concentration, motility, morphology, vitality and sperm chromatin integrity by AO binding.

Statistical analysis

Values were expressed as means ± standard deviation (SD). Statistical significance was assessed using student's *t* test. *P* values of less than 0.05 were considered statistically significant.

Results

The ability of the CO_2 and the CO_2-free systems for the sperm extraction by swim up technique was compared using twenty five semen samples. Since the same semen sample was used for comparison, the two groups were homogenous for concentration, motility, viability, morphology and DNA integrity.

The semen sample tested had a concentration of 48.8±30.32 millions per milliliter, a mean sperm motility of 65.53±9.92, mean sperm morphology of 31.33±5.2, viability of 66.26±7.98 and a mean percentage of sperm exhibiting denatured DNA of 17.94±6.23.

Although, the sperm recovery was approximately 50% of the initial sperm concentration, there was no statistically significant difference in the sperm count between the CO_2 and the CO_2-free systems (Figure 1A). Similarly, no significant differences were found in post wash motility (Figure 1B) and viability (Figure 1C) between the two study groups.

Sperm morphology was also assessed for the evaluation of two systems. Although, the number of sperm recovered using the CO_2-free system had slightly higher abnormal forms compared to the CO_2 system, the differences were statistically insignificant (Figure 1D). Since there is no report on the influence of incubation in bicarbonate buffer alone +CO_2 or in bicarbonate+HEPES+water bath on the sperm chromatin structures, we used AO binding ability to assess the incidence of chromatin denaturation in the sperm recovered from these systems. Orange-red fluorescence is an indication of presence of single stranded DNA and the sperms with

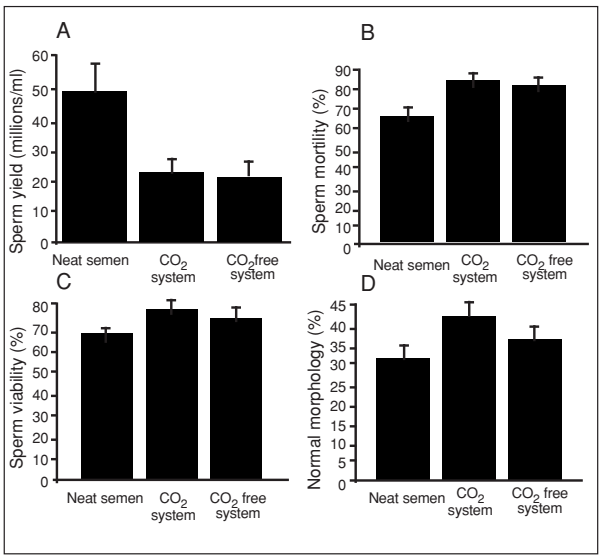

Figure 1. Comparison of CO_2 and CO_2 free incubation systems on; A) sperm yield; B) extraction of number of motile sperm; C) number of viable sperm; D) morphologically normal sperm.

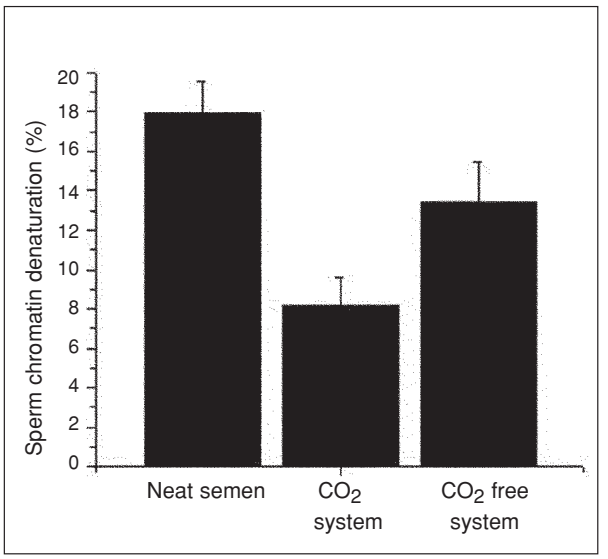

Figure 2. Sperm chromatin denaturation in the spermatozoa before and after sperm preparation using CO_2 and CO_2 free systems.

native DNA fluoresce greenish. The number of sperms with denatured DNA in the pre-wash sample was approximately 18%. Although, both the CO_2 and the CO_2-free systems eliminated the number of sperms with denatured DNA significantly ($p<0.01$), there was no statistically significant difference between two methods in extracting chromatin intact spermatozoa. However, the number of denatured sperm was slightly higher in the CO_2-free system (Figure 2).

Discussion

Our results demonstrated no significant difference between the CO_2 and the CO_2-free systems for sperm extraction from normozoospermic men as measured by sperm yield, motility, viability and percentage normal morphology. Analysis of the chromatin denaturation showed that the mean percentages were not significantly different between the two preparation methods but chromatin denaturation in the spermatozoa extracted by the CO_2-free system was higher than that prepared by the CO_2 system. Although, the unwashed sample had higher number of sperm with denatured chromatin, both methods significantly eliminated denatured spermatozoa. Assessment of DNA integrity after any preparatory method has been limited to date. However, the swim up technique is effective in eliminating morphologically abnormal with damaged DNA sperm when compared to other techniques (12,13). Hence, we used swim up technique to compare the effectiveness of two systems proposed in this study.

The water bath system is not usually being used in andrology for sperm separation method. The one reason is that most of the culture media available commercially for sperm separation are designed for CO_2 systems for maintaining the optimum pH and these media are not suitable for maintaining pH in a closed system like water bath. We have used HEPES, an organic pH buffer at a concentration of 15 mM in combination with bicarbonate buffer to maintain the pH at 7.4-7.6 for CO_2 free system. Since, earlier reports have shown that a concentration of 25 mM HEPES in the embryo culture medium is detrimental to bovine embryonic development (14), we have used only 15 mM HEPES in combination with bicarbonate buffer in our study which is the acceptable level for the medium used in the ICSI procedure. Bicarbonate is not required for acrosomal exocytosis but it is essential for capacitation, exerting roles beyond its action as pH buffer (15); hence, the retention of bicarbonate for CO_2 free system in our study. Furthermore, it has been reported that inclusion of HEPES in bicarbonate-containing medium during gamete co-incubation did not affect fertilization, showing that HEPES does not exert an inhibitory effect (16). In addition, several recent reports have shown that processing of semen and incubation of sperm in protein supplemented with HEPES best preserved sperm motility and vitality at room temperature (17,18).

The costs of infertility evaluation and treatment are frequently passed directly to the patient because of limited insurance coverage and high expenses towards procurement and maintenance of the equipments. Hence, a detailed study is necessary to identify the cost-effective approaches of certain diagnostic procedures in infertility treatments. In this context, the water bath could serve as an ideal alternative to carbon dioxide incubator for the people who would like to begin with only intrauterine insemination program with minimum investment on equipments. Water bath is less expensive and maintenance cost is significantly lower than the CO_2 incubator. In addition, the temperature distribution is more rapid and uniform within the water chamber. The andrology laboratories which perform trial sperm wash as a part of diagnosis can also use water bath for sperm extraction as we have found no difference in the sperm yield between two methods. Ultimately, pregnancy

rates are the key issue in any medically assisted conception program. Therefore, further studies are required to document the superiority of CO_2-free incubation system in achieving success in IUI program.

Acknowledgement
Authors are thankful to Jayalaxmi Pai and Sandhya Patil for their excellent technical help. This work was supported by research grant from the Kasturba Medical College, Manipal.

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