Bali Medical Journal (Bali Med. J.) 2014, Volume 3, Number 3: 129-132
P-ISSN.2089-1180, E-ISSN.2302-2914

THE ADDITION OF CAFFEINE IN EARLE'S BALANCED SALT SOLUTION MEDIA WITH WASHING UP METHOD INCREASE MEMBRANE INTEGRITY AND ACROSOMAL SPERM

Bagus Komang Satriyasa and Agung Nova Mahendra

Departement of Pharmacology, Faculty of Medicine, Udayana University, Bali-Indonesia

Background: caffeine, a methylxanthine derivate, appears to inhibit phosphodiesterase, thereby inhibiting the break down of cAMP and increasing its concentration inside cell. This study aims to assess the effect of caffeine addition in Earles's Balanced Salt Solution (EBSS) on the increase in membrane integrity and acrosome reaction of spermatozoa using swim up method. **Methods:** This study was carried out at the Clinic of Sexology and Andrology, Sanglah Public Hospital at Denpasar Bali-Indonesia. This study was an experimental study using the design of pre and post test paired control group design. The samples were sperm specimens of eighteen infertile couple male or volunteers who were infertile with age ranged between 20-40 years old. The samples were divided into two groups: treatment group (caffeine + EBSS) and control group (EBSS). The data were analysed statistically by normality test (Kolmogorov - Smirnov Goodness of Fit Test), Homogeneity test, and Paired Student's t test. **Results:** The results showed that the caffeine addition in EBSS medium could increase significantly ($p<0.05$). The integrity of the sperm membrane obtained were from 81.30 % to 86.60 % and acrosomal reaction from 82.60% to 89.60% evaluated by hypo-osmotic swelling test (HOS). The conclusion of this study is that addition of caffeine in EBSS medium increases significantly membrane integrity and acrosomal reaction of the human sperm.

Keywords: EBSS; caffeine; membrane integrity; and acrosomal reaction of sperm

INTRODUCTION

Sperm preparation should be carried out before aplying the Assisted Reproductive Technology (ART).[1,2] Some commonly known sperm preparation methods in Indonesia are swim up washing method (SUM), density gradient centrifugation (DGC), and side migration technique (SMT) (TMS). SUM technique is a favourite technique elaborated in many andrology clinics and the most commonly used medium in this process is Earli's Balance Salt Solution (EBSS). In ART, pregnancy rate is relatively low reaching only 5% per cycle in IUI and 8% in IVF.[2] The cause of low achievement is multifactorial in nature. Some of the causative factors are the failure of spermatozoa to adhere onto the ovarial zona ifferen and the failure of acrosomal reaction to occur. The poor binding of ifference to ovum is commonly caused by defect in ifference membrane.[2,3] Substances or drugs is needed to enhance ifference membrane integrity and acrosomal reaction, which ultimately lead to improved ifference property of the difference. One of them is caffeine (1,3,7-trimethyl-2, 6-dioxy-purine). Caffeine, a phosphodiesterase inhibitor (PDEI) boosts ifference metabolic activity. Caffeine increases ifference motility, membrane integrity, and acrosomal reaction rate in vitro.[4,5] Khalili et al (1999) reported that membrane integrity of caffeine-washed spermatozoa is higher than those washed using standard medium (33,33% versus 27%).[6] Moreover, Matas et al (2002) reported that hypoosmotic swelling (HOS) test showed significant difference ($p < 0,01$) between sodium benzoate caffeine-treated spermatozoa and the control.[7]

In this study, SUM technique was apllied, in order to study the effect of caffeine addition into EBSS media on the ifference membrane integrity and acrosomal reaction. The hypothesis of this study was the caffeine addition into EBSS media increases the membrane integrity and acrosomal reaction of SUM-harvested spermatozoa.

Address of Correspondence:
Bagus Komang Satriyasa
Departement of Pharmacology, Faculty of Medicine, Udayana University Bali-Indonesia
Email: bsatriyasa@gmail.com

RESEARCH METHODS

The study was an experimental study with Pre-and post-test paired control group design. The sample counting was independent with average

difference as a test of difference significance based on Colton's formula.[8] Calculation using the formula, the minimal number of the sample obtained was 18 samples. In this study, we used 20 human samples.

Samples are recruited from patients or volunteers who came to Andrology and Sexology Polyclinic, Sanglah General Hospital, Bali-Indonesia, after sexual abstinence for 2-7 days, and had been diagnosed with asthenozoospermia. Early examination was done, and samples who meet the inclusion criteria were recruited into this study. The individual samples were divided into 2 groups; control group (their spermatozoa were washed with EBSS) and the treatment group (their spermatozoa were washed with EBSS + caffeine). Spermatozoa were obtained from masturbation the sperma had been transferred to the polyclinic in less than 1 hour after ejaculation. The ejaculates were contained within wide-mouthed glass placed in room temperature 37 ^{0} C and immediately examined after perfect liquefaction took place. The ejaculate volume per sample has to be more than 2 cc, with minimum concentration of 5 x 10^6 spermatozoa/cc as prerequisites for SUM application.[9]

Sperm washing method was as follow: EBSS medium (2 cc) of was poured into centrifuge tube. One cc of ifferenc-ejaculate was then added into the tube and mixed thoroughly. This sample was centrifuged for 10 minutes in low velocity (300g). Supernatant was poured away, add 2 cc of EBSS medium, and centrifuged in the same manner with the first centrifugation. The resultant supernatant must be poured away again. We poured down 1 cc of EBSS medium carefully onto the precipitate and incubated the mixture for 60 minutes. It was done to give a chance to spermatozoa with good membrane integrity to reach the mixture surface. After being incubated, about 2/3 of the upper part of the mixture was examined for ifference membrane integrity and acrosomal reaction.[10] This procedure was also applied to ejaculate samples treated with caffeine-fortified EBSS medium. Acrosomal reaction examination was based on the dual staining technique developed by Susilawati (1999) such as follow: sperm suspension aliquot (10-20 µl) was smeared on an object glass and dried in 40 ^{0}C. The smear was then stained with 10 % Giemsa stain in distilled water for 35-40 minutes, washed under the water faucet, and observed using light microscope. The colour of acrosome was dark pink, meanwhile acrosome-absence spermatozoa could not catch up the stain.[11]

HOS test procedure was as follow: one cc of larutan pembangkak freezed in closed Eppendorf tube in 37^{0}C for 5 minutes, and then post-liquefaction ejaculate was added. We mixed this mixture gently and leave it in 37^{0}C for 30 minutes. The mixture was then examined under light microscope. The parameters observed were phase difference and ifference score (based on tail structural transformation per 100 spermatozoa.[10]

RESULTS

The data obtained from the spermatozoa of treatment and control group before treatment were normally-distributed (p>0.05).[11]

Harvested Sperm Samples after Treatment

The observed sperm variables after treatment were membrane integrity (HOS test) and acrosomal reaction. Data distribution was tested with Kolmogrov-Smirnov test, in which the results can be seen in Table 1.

Table 1
Data Normality of SUM-harvested spermatozoa in EBSS dan EBSS + Caffeine Media

Harvest	N	Mean	SD	K-SZ	p
Host tests (%)					
EBSS	20	81.30	10.21	1.02	0.25
EBSS + caffeine	20	86.60	9.93	1.05	0.22
Acrosomal reaction					
EBSS	20	82.60	5.64	0.54	0.93
EBSS + caffeine	20	89.60	4.90	0.55	0.92

It can be clearly seen in Table 1 that the data on membrane integrity and acrosomal reaction in control and experiment group were in normal distribution (p>0.05).

The Enhancement of Membrane Integrity after Treatment

Paired t-test was elaborated in the statistical analysis of HOS test results of control and treatment group after treatment. The data distribution was normal. The results of analysis can be seen in Table 2.

Table 2
The HOS test results in EBSS and EBSS + caffeine-washed spermatozoa

Parameters	Pre	Post	Mean Increase (%)	p
EBSS (control	76.60	81.30	4.70 (2.54)	0.001
EBSS + caffeine (treatment)	76.60	86.60	10 (3.58)	0.001

Improvement of HOS test results of spermatozoa following caffeine addition into EBSS medium were significantly higher than those treated with EBSS medium only (p<0.05).

The Difference of Improved Membrane Integrity after Treatment

The difference of ifference membrane integrity data (shown as HOS test results) between

group was analyzed by elaborating the parametric paired t-test, which can be seen in Table 3.

Table 3
HOS test results ifference between groups after treatment

Parameters	Average score (%)	Average different (%)	SD	*t*	*p*
EBSS (control	81.30	5.30	1.98	11.99	0.001
EBSS + caffeine (treatment)	86.60				

SD = standard deviation

The HOS test results between groups after treatment were significantly different, in which that HOS test results in the treatment group were significantly higher than those of control group.

The Difference of Acrosomal Reaction Improvement after Treatment

Acrosomal reactions in control and treatment group after treatment were expressed as percentage and can be seen in Table 4.

Table 4
The difference of acrosomal reaction improvement after treatment between groups

Parameters	Average score (%)	Average different (%)	SD	*t*	*p*
EBSS (control	82.60	7.00	2.13	14.71	0.001
EBSS + caffeine (treatment)	89.60				

SD = standard deviation

Statistical analysis showed that acrosomal reactions between groups differ significantly ($p<0.05$). Thus, caffeine addition into EBSS medium increases the number of acrosomal reactions significantly.

DISCUSSION

Caffeine Improves PRA-harvested Spermatozoal Membrane Integrity

In this study, the caffeine-related increase in membrane integrity was indicated by the increase in HOS test results. The HOS of control group was increased from 76.60 % to 81.30 %, meanwhile, in the treatment group was increased from 76.60 % to 86.60 %. Caffeine addition to the EBSS medium contributed to the extra of 5.30% result in HOS test compared to the control group result. Khalili et al. (1999) found that the membrane integrity of caffeine-washed spermatozoa was higher (33.33%) than those washed using standard medium (27 %).[6] Matas et al. (2002) also reported a significant HOS test results difference ($p < 0.01$) between caffeine-sodium benzoate-washed spermatozoa and the spermatozoa in control group.[7]

The increase in membrane integrity is attributable to the inhibitory effect of caffeine on PDE. The PDE inhibition elevates intracellular cAMP level that increases glycolysis, ATP synthesis, enzymatic activity, muscle contraction and relaxation, and also protein synthesis and secretion. Caffeine-related PDE inhibition stimulates the inrease of ATP-ase activity in spermatozoal cell membrane. This enzyme is located in the midpiece of spermatozoa, and maintains the internal homeostasis of sodium and potassium ions. Elevated ATP-ase activity can alter the spermatozoal membrane permeability.[6]

Membrane integrity improvent is associated with the increase of membrane permeability and transport of nutrients which are essential for optimal metabolisme. In accordance with this theory, Jeyendra et al. (1986) stated that spermatozoal membrane permeability is associated with spermatozoal.[12]

Acrosomal Reaction is enhanced by Caffeine Addition into EBSS Medium

In this study, the number of acrosomal reaction was 82.60% in control group, meanwhile acrosomal reaction was observed in 89.60% of the sample in the treatment group. Thus, it can be seen clearly that caffeine addition into EBSS medium increases acrosomal reaction significantly.

Kim et al (1990) stated that caffeine addition could increase fertilization success in terms of increased capacitation and acrosomal reaction.[13] Moreover, Park et al (1989) found that caffeine and heparin works synergistically to enhance the initiation of capacitation and acrosomal reaction.[14] These findings were apllied by Nakao ans Nakatsuji (1990) in the preaparation of spermatozoa with good quality.[15] They washed the spermatozoa using Brackett and Oliphant (BO) medium without Bovine Serum Albumin (BSA). Instead, they use 10 mM of caffeine as a substitute for BSA.[15]

Cosciani et al. (2001) studied the effect of caffeine and calcium administration on bovine embryo development *in vitro*.[16] They found that caffeine could increase acrosomal reaction to 53%, relative to the respective control. It was also reported that the average cleavage rate after PRA application bovine embryo growth did not differ significantly between group treated with caffeine and calcium. Funahashi and Nagia (2001) reported that caffeine and adenosine could increase wild boar spermatozoal acrosomal reaction and penetration rate.[17] Penetration rate in caffeine-treated group, adenosine-treated group, and control group was 99.1 ± 0.9, 72.4 ± 2.0 %, and 54.8 ± 5.1 %, respectively.

Pereira et al (2000) found that caffeine, calcium, or heparin could stimulate the initiation of bovine spermatozoal acrosomal *in vitro*.[18] Moreover, Baldi et al (1996), found that PDEIs

such as caffeine, pentoxyfilin, and progesterone could also induce acrosomal reaction *in vitro*.[4]

Caffeine increases acrosomal reaction by inhibiting PDE. The PDE inhibition will increase intracellular cAMP concentration. These events will in turn enhance glycolysis and ATP biogenesis that lead to increased enzyme activity, muscle contraction and relaxation, protein synthesis and secretion.[6]

Increased cAMP level activates cAMP-dependent kinase (PKA), which in turn increases protein phosphorylation, and induces acrosomal reaction to take place. Increased cAMP level can also increase spermatozoal membrane permeability to ions, in which Na^+ and Ca^{2+} ions transports into intracellular space and ion H^+ into extracellular space are enhanced. These events increase intracellular pH that will ultimately lead to the occurence of acrosomal reaction.[4]

It can be conluded that caffeine addition into EBSS media could promote significant increases in membrane integrity and acrosomal reaction of spermatozoa.

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