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Research Article

Drone Semen Cryopreservation with Protein Supplemented TL-Hepes Based Extender [1]

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Abstract

The aim of the current study was to determine the optimum concentration of bovine serum albumin for post-thawing quality of drone sperm and this is the first study to evaluate the effect of BSA supplemented TL-Hepes based extenders for drone semen cryopreservation. Sexually mature drones were used for semen collection. Pooled semen was diluted with TL-Hepes based extender supplemented with different concentrations of BSA (1 mg/mL, 3 mg/mL, and 5 mg/mL) or without BSA (control), at a final concentration of 100×10^6 spermatozoon/mL. Motility, plasma membrane functional integrity (HOST), and defected acrosome (PSA-FITC) were evaluated in the study. At post thaw, the highest sperm motility rates were obtained in the BSA5 group (P<0.05). Functional integrity of sperm membrane was better preserved in the BSA3 and BSA5 groups compared to the other groups. The acrosomal integrity rates were higher in BSA5 group than in control group (P<0.05). The study shows that bovine serum albumin supplemented TL-Hepes based extenders have beneficial effect on drone semen parameters at post-thaw. The results of the study demonstrated a notable advantage of using 5 mg/mL of BSA in TL-Hepes based extender.

Keywords: *Drone spermatozoa, Bovine serum albumin, Cryopreservation*

Arı Spermasının Protein Eklenmiş TL-Hepes Bazlı Sulandırıcı İle Dondurulması

Öz

Bu çalışmanın amacı, arı spermasının çözdürme sonrası kalitesi için en uygun sığır serum albümin konsantrasyonunu belirlemekti ve bu çalışma, arı spermasının dondurulması için BSA eklenmiş TL-Hepes bazlı sulandırıcıların etkisini değerlendiren ilk çalışmadır. Cinsel olarak olgun arılar sperma toplanması için kullanıldı. Alınan spermalar bir araya getirildikten sonra final konsantrasyonu 100×10^6 spermatozoon/mL olacak şekilde farklı dozlarda BSA içeren (1 mg/mL, 3 mg/mL, ve 5 mg/mL) ve içermeyen (kontrol) TL-Hepes bazlı sulandırıcılarla sulandırılmıştır. Spermanın değerlendirilmesi amacıyla motilite, plazma membran fonksiyonel bütünlüğü (HOST), ve akrozomal bozukluk (PSA-FITC) değerlerine bakılmıştır. Eritme sonrası en yüksek motilite oranları BSA5 grubunda elde edilmiştir (P<0.05). BSA3 ve BSA5 gruplarında fonksiyonel membran bütünlüğü diğer gruplara göre daha iyi korunmuştur. Akrozomal bütünlük oranları BSA5 grubunda kontrol grubuna göre daha yüksek bulunmuştur (P<0.05). Çalışma sonucunda, sığır serum albümini eklenmiş TL-Hepes bazlı sulandırıcıların eritme sonrası arı sperm parametreleri üzerinde yararlı etkisi olduğu görülmektedir. Çalışma sonucu elde edilen veriler göz önüne alındığında TL-Hepes bazlı sulandırıcıya 5 mg/mL BSA ilavesinin önemli bir avantaj oluşturduğu görülmektedir.

Anahtar sözcükler: *Arı sperması, Sığır serum albumini, Kriyoprezervasyon*

INTRODUCTION

Cryopreservation is the pillar of the reproductive biotechnology [1]. This reversible process brings sperm metabolism to a standstill, in this way the genetic materials are successfully storaged for a long time [2]. Although

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cryopreservation is a reversible operation, there are some harmful effects (cold shock, ice crystallization, lipid peroxidation etc.) on spermatozoa [2,3]. These adverse effects may cause irreversible decreases on motility, viability and fertilization ability of spermatozoon [4,5]. Therefore, the achievement of sperm cryopreservation depends on minimizing the adverse effects and maintaining the post-thaw semen quality [1,5].

Freezing of drone semen will improve preservation of the genetic diversity in the honey bee in different regions. For this purpose, genetic diversity of honey bee colony genetic diversity may be explored for the disease resistances [6-10]. Drone semen cryopreservation has been accomplished in recent years. However, the freezing success of drone semen has not reached the desired level nowadays [11,12].

Bovine serum albumin (BSA) has a multifunctional effect on spermatozoa with its macromolecular structure and antioxidant capacity. Therefore, BSA increases the post-thaw sperm motility and protects the plasma membrane against cold shock [13]. In addition, BSA increases the possibility of sperm-zona pellucida interactions and fertility results. For these reasons, various extenders supplemented with BSA are being used for cryopreservation or liquid storage of bull [14], ram [15], goat [16], stallion [17], buffalo [18] and rabbit semen [19]. However, the effect of BSA supplemented TL-Hepes based extender on drone semen cryopreservation has not been examined until now.

The drone semen cryopreservation contributes to the selection and conservation of gene lines in superior genetic characteristics [8,9]. For this purpose, freezing and storage of drone semen which has superior genetic characteristics, allows preservation and widespread of the specified characteristics. The study was conducted to compare the various concentrations of BSA supplemented extenders to freeze of drone spermatozoa.

MATERIAL and METHODS

The chemicals were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) in the study.

Experimental Design

The study was designed to investigate the efficacy of BSA supplementation to the extender in drone semen cryopreservation. Therefore, different concentrations of BSA (0 mg/mL, 1 mg/mL, 3 mg/mL or 5 mg/mL) supplemented TL-Hepes based extender was used for post-thaw drone semen quality.

Semen Extender Preparation

Hepes based extenders contained 114 mmol NaCl, 3.2 mmol KCl, 2.0 mmol $CaCl_2 2H_2O$, 0.5 mmol $MgCl_2.6H_2O$, 25.0 mmol $NaHCO_3$, 0.40 mmol $NaH_2PO_4{\cdot}H_2O$, 10 mmol NaLactate (60% syrup), 200 µL Catalase, 10% DMSO, 4 g/L penicillin G, 3 g/L dihydrostreptomycin in distilled water. BSA added to each group of extenders according to experimental design.

Semen Collection and Dilution

Strong and healthy colonies were used to produce honey bee drones that were collected from 5 colonies. Sexually mature drones (15 days or more) were used for sperm collection. Ejaculation was triggered by exerting pressure on the thorax and then gently squeezing the abdomen [12]. Drone semen was taken up into the Schley syringe under a stereo microscope. The collected semen volume per drone was approximately 1 µL in the experiment. The drone semen was pooled (five times) in order to eliminate individual differences. The average volume of each pooled semen was 100 µL.

Each pooled semen were divided into five equal aliquots and diluted separately for a final concentration of approximately $100x10^6$ (spermatozoon/mL) with control or BSA supplemented TL-Hepes based extenders. Diluted samples were cooled to 5°C in an h. After cooling, the sperm samples were equilibrated for further 120 min at 5°C.

Semen Freezing and Thawing

Equilibrated semen was placed into straws (0.25 mL) and frozen in liquid nitrogen vapor. The straws were stored in a liquid nitrogen tank. Three straws were used for post-thaw semen parameters in each group.

Semen Evaluation

Semen evaluation was carried out via thawing the straws in a water bath that has 37°C warmth. Sperm cells were assessed for motility, functional integrity of the cell membrane (hypoosmotic swelling test) and the integrity of the acrosomes with FITC conjugated Pisumsativum agglutinin. The same person conducted the processes and measurements along the research. Semen motility was determined using a phase-contrast microscope (Nikon Alphaphot YS, Japan) (400×) with a warm slide that heated to 37°C and the motility results were expressed in percent [12].

The hypoosmotic swelling test (HOST) was assessed based on coiled tails at drone semen. Semen (10 µL) was incubated with host solution (100 µL of 100 mOsm) at 37°C for 30 min. At least 200 sperm cells were evaluated and spermatozoa with coiled tail were recorded [20].

Acrosomal integrity was evaluated with using Fluorescein lectin staining assay (PSA-FITC). Briefly, spermatozoa (10 µL) were suspended in 100 mL phosphate buffered saline (PBS) and centrifuged at 100 RCF (g) for 5 min. The sperm pellet was resuspended in 100 mL PBS. Spermatozoa were smeared on glass microscope slides using another slides and fixed with acetone at 4°C for 15 min. Spermatozoa were stained with the solution of PSA-FITC in a dark chamber at

37°C for 1 h. At least 200 drone spermatozoa were assessed at per smearunder a fluorescence microscope [21].

Statistical Analysis

The results were analyzed using SPSS (SPSS 23.0 for Windows; SPSS, Chicago, IL, USA) and presented as mean ± standard deviation. Shapiro Wilk test was used as normality test. Semen parameters were analyzed using one-way ANOVA followed by Tukey. Pearson correlation coefficient was used to evaluate the relationships among the values of motility, plasma membrane functional integrity and acrosomal integrity. $P<0.05$ were considered to be statistically significant.

RESULTS

Sperm motility, plasma membrane functional integrity and defected acrosome rate of pooled semen were 88.00±2.73%, 89.40±3.36% and 6.40±1.67%, respectively. The percentages of sperm motility, plasma membrane functional integrity and defected acrosome of post-thawed drone semen from BSA and control groups, were indicated in *Table 1*.

Motility of drone spermatozoon was progressively decreased through the process of freeze-thawing ($P<0.001$). The motility rates better preserved in the BSA groups than the control groups ($P<0.05$). The highest percentage of motility was obtained in BSA5 group at post-thaw ($P<0.05$).

Plasma membrane functional integrity *(Fig. 1)* rates were reduced after thawing procedure ($P<0.001$). BSA3 and BSA5 groups had better results than BSA1 and control groups in terms of the functional integrity of the cell membrane ($P<0.05$).

Sperm acrosome *(Fig. 2)* was negatively affected by the freeze-thaw process ($P<0.001$). The percentage of defected acrosome in BSA5 group was lower than control group ($P<0.05$). Defected acrosome rates were not found significant among BSA groups ($P>0.05$).

The Pearson correlation test values are shown in *Table 2*. Motility was positively correlated with membrane integrity but negatively correlated with defected acrosome rates ($P<0.01$). In addition, there was a negative correlation between membrane integrity and defected acrosome rates ($P<0.01$).

DISCUSSION

Evidences suggest that, cryopreservation has a destructive effect on spermatozoon because of temperature change, cold shock and ice crystallization. These adverse effects provoke to decrease of motility, acrosomal integrity, and

Table 1. *The mean results of studied sperm post-thaw parameters on different extender groups*

Group	Motility (%)	HOST (%)	Defected Acrosome (%)
Control	39.33±3.71^{a}	59.20±3.58^{a}	24.20±3.82^{a}
BSA1	43.66±4.41^{b}	61.53±4.15^{a}	21.34±2.79^{ab}
BSA3	50.00±4.62^{c}	66.06±3.15^{b}	21.17±2.97^{ab}
BSA5	54.33±3.71^{d}	67.73±4.07^{b}	18.80±3.44^{b}

Data is presented in Mean± S.D.
^{a,b,c,d} Values with different superscripts in the same column are significantly different (P<0.05)

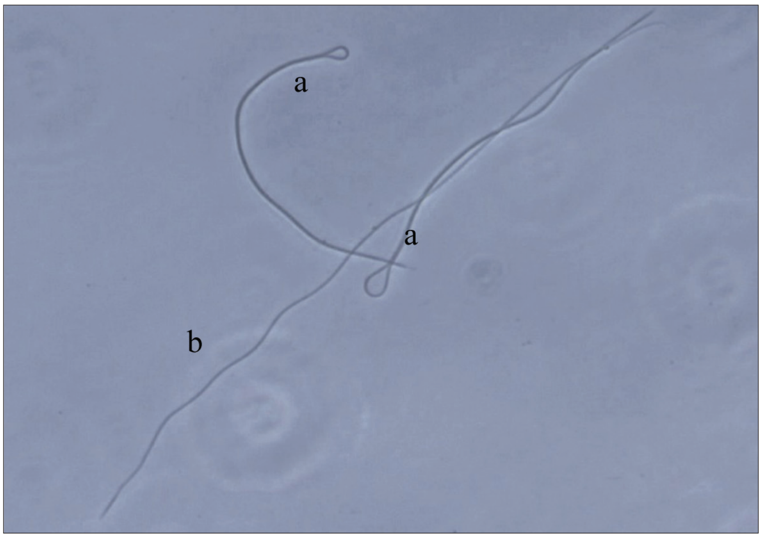

Fig 1. Membrane integrity (a) and damaged membrane (b) by HOS test

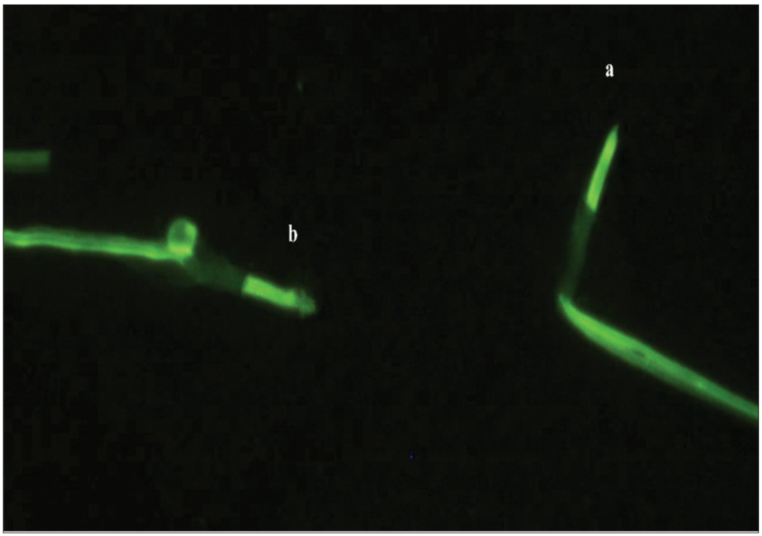

Fig 2. FITC-PSA stained spermatozoa with intact (a) and defected acrosome (b)

Table 2. *Correlation coefficient (r) of studied drone semen parameters*

Spermatological Parameters	HOST (%)	Defected Acrosome (%)
Motility	0.797**	-0.801**
Host (%)		-0.762**

*** Significance of the correlation is at the P<0.01*

fertilizing ability of spermatozoa [5,21-23]. Various extenders were used to minimize the adverse effect of freeze thaw process [5,8,22-24]. In the current study, we compared the effect of exogenous addition of BSA in TL-Hepes based extender on drone semen quality at post thaw. This is the first study to evaluate the effect of BSA supplemented TL-Hepes based extenders for drone semen cryopreservation.

After artificial insemination of queen bees, only motile spermatozoa could arrive to the spermatheca over a 48 h period [24,25]. In the study, BSA5 group yielded higher motility rates than other groups at post thaw time point (P<0.05). The post-thaw motility values of drone semen cryopreserved with different extenders ranged between 25%-62% [8,11,12,24]. After thawing, the motility rate in BSA5 group has a common point with the findings of other studies. In the study; BSA supplementation prompted to clear increase on motility at post-thaw. In addition, increasing doses of BSA positively affected sperm motility.

Plasma membranes have an important role in spermato-zoon metabolism [19]. Therefore, integrity of the plasma membrane is essential for capacitation, acrosome reaction and oocyte fusion of sperm [26]. However, plasma membrane could lose its selective permeability because of the cold shock [27]. The protection against cold shock is possible with increasing the fluidity of cell membrane [28]. The protective effect of BSA against cold shock is based on this expected impact. BSA attaches to the sperm membrane and then changes sperm membrane lipid composition and decreases to phospholipid concentration [29]. In our study, the HOST rates in BSA3 and BSA5 group were higher than in the other groups (P<0.05). The HOST values have common points for the results with the previous research [12].

Acrosomal integrity is related with sperm penetration and fusion to zona pellucida. Therefore, this is another important factor in the fertilization process. The other adverse effect of cryopreservation is the acrosomal damage [5,21,23]. Bovine serum albumine succesfully protects the integrity of acrosome [15,19]. In the study, there was no statistical difference among BSA groups. Additionaly, BSA5 group preserved acrosomal integrity better than control group. The statistical difference of acrosome integrity between the groups of control and BSA5 by evaluating with PSA-FITC staining assay will brighten the path of further studies for this area.

In the study, a positive correlation was obtained between the post thaw sperm motility and plasma membrane functional integrity rates (P<0.01). As this is an expected result; the motility partly depends on the transport of compounds across the cell membranes [23], there are previous reports with similar findings [5]. In addition, motility negatively correlated with non-intact acrosome rates (P<0.01). These results showed the same understanding that pointed with the other reports [20].

The outcomes of the study indicated that BSA5 group preserved sperm motility better than other groups. Considering to all sperm parameters (motility, plasma membrane functional integrity and acrosomal integrity); BSA5 group was the optimum for drone semen cryopreservation among studied doses. Beneficial effect of BSA supplementation looked promising to increase the utility of TL-Hepes based extender for drone spermatozoa. Further studies should take place to improve the effects of the BSA supplemented TL-Hepes based extender concerning with sperm fertility.

REFERENCES

1. Salamon S, Maxwell WMC: Storage of ram semen. *Anim Reprod Sci,* 62, 77-111, 2000. DOI: 10.1016/S0378-4320(00)00155-X

2. Holt WV, Penfold LM: Fundamental and practical aspects of semen cryopreservation. **In,** Chenoweth P, Lorton S (Eds): Animal andrology: Theories and Applications. 76-99, CAB International CABI, Wallingford Oxfordshire, 2014. DOI: 10.1079/9781780643168.0076

3. Fiser PS, Fairfull RW: The effects of rapid cooling (cold shock) of ram semen, photoperiod, and egg yolk in diluents on the survival of spermatozoa before and after freezing. *Cryobiology,* 23, 518-524, 1986. DOI: 10.1016/0011-2240(86)90061-1

4. O'Connell M, McClure N, Lewis SEM: The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Hum Reprod*, 17 (3): 704-709, 2002. DOI: 10.1093/humrep/17.3.704

5. Ustuner B, Alcay S, Toker MB, Nur Z, Gokce E, Sonat FA, Gul Z, Duman M, Ceniz C, Uslu A, Sagirkaya H, Soylu MK: Effect of rainbow trout *(Oncorhynchus mykiss)* seminal plasma on the post-thaw quality of ram semen cryopreserved in a soybean lecithin-based or egg yolk-based extender. *Anim Reprod Sci*, 164, 97-104, 2016. DOI: 10.1016/J.ANIREPROSCI.2015.11.017

6. Seeley TD, Tarpy DR: Queen promiscuity lowers disease within honeybee colonies. *Proc Biol Sci*, 274, 67-72, 2006. DOI: 10.1098/rspb.2006.3702

7. Mattilla HR, Seeley TD: Genetic diversity in honey bee colonies enhances productivity and fitness. *Science*, 317, 362-364, 2007. DOI: 10.1126/science.1143046

8. Taylor MA, Guzmán-Novoa E, Morfin N, Buhr MM: Improving viability of cryopreserved honey bee *(Apis mellifera* L.) sperm with selected diluents, cryoprotectants, and semen dilution ratios. *Theriogenology*, 72, 149-159, 2009. DOI: 10.1016/j.theriogenology.2009.02.012

9. Hopkins BK, Herr C: Factors affecting the successful cryopreservation of honey bee *(Apis mellifera)* spermatozoa. *Apidologie*, 41, 548-556, 2010. DOI: 10.1051/apido/20010006

10. Hopkins BK, Herr C, Sheppard WS: Sequential generations of honey bee *(Apis mellifera)* queens produced using cryopreserved semen. *Reprod Fertil Dev,* 24, 1079-1083, 2012. DOI: 10.1071/RD11088

11. Wegener J, May T, Kamp G, Bienefeld K: A successfull new approach to honeybee semen cryopreservation. *Cryobiology*, 69, 236-242, 2014. DOI: 10.1016/j.cryobiol.2014.07.011

12. Alcay S, Ustuner B, Cakmak I, Cakmal S, Nur Z: Effects of various cryoprotective agents on post thaw drone semen quality. *Kafkas Univ Vet Fak Derg,* 21, 31-35, 2015. DOI: 10.9775/KVFD.2014.11515

13. Uysal O, Bucak MN: Effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. *Acta Vet Brno*, 76, 383-390, 2007. DOI: 10.2754/avb200776030383

14. Uysal O, Bucak MN, Yavas I, Varisli O: Effect of various antioxidants on the quality of frozen-thawed bull semen. *J Anim Vet Adv*, 6, 1362-1366, 2007.

15. Gokce E, Alcay S, Gul Z: Positive effect of BSA supplemented soybean lecithin based extender on liquid storage of ram semen at 5ºC. *Ankara Univ Vet Fak Derg,* 64, 313-320, 2017.

16. Amidi F, Farshad A, Khor AK: Effects of cholesterol-loaded cyclodextrin during freezing step of cryopreservation with TCGY extender containing bovine serum albumin on quality of goat spermatozoa. *Cryobiology*, 61, 94-99, 2010. DOI: 10.1016/j.cryobiol.2010.05.006

17. Ball BA, Medina V, Gravance CG, Baumbe J: Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. *Theriogenology*, 56, 577-589, 2001. DOI: 10.1016/S0093-691X(01)00590-8

18. Akhter S, Rakha BA, Iqbal R, Ansari MS: Effect of bovine serum albumin on motility, plasmalemma, viability and chromatin ıntegrity of buffalo bull spermatozoa. *Pak J Zool*, 46, 115-120, 2014.

19. Sariozkan S, Turk G, Canturk F, Yay A, Eken A, Akcay A: The effect of bovine serum albumin and fetal calf serum on sperm quality, DNA fragmentation and lipid peroxidation of the liquid stored rabbit semen. *Cryobiology*, 67, 1-6, 2013. DOI: 10.1016/j.cryobiol.2013.04.002

20. Alcay S, Toker MB, Onder NT, Gokce E: Royal jelly supplemented soybean lecithin-based extenders improve post-thaw quality and incubation resilience of goat spermatozoa. *Cryobiology*, 74, 81-85, 2017. DOI: 10.1016/j.cryobiol.2016.11.011

21. Nur Z, Zik B, Ustuner B, Sagirkaya H, Ozguden CG: Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. *Theriogenology*, 73, 1267-1275, 2010. DOI: 10.1016/j.theriogenology.2009.12.007

22. Bucak MN, Sariozkan S, Tuncer PB, Sakin F, Atessahin A, Kulaksiz R, Cevik M: The effect of antioxidants on post-thawed Angora goat *(Capra hircus ancryrensis)* sperm parameters, lipid peroxidation and antioxidant activities. *Small Ruminant Res,* 89, 24-30, 2010. DOI: 10.1016/j.smallrumres.2009.11.015

23. Alcay S, Toker MB, Gokce E, Ustuner B, Onder NT, Sagırkaya H, Nur Z, Soylu MK: Successful ram semen cryopreservation with lyophilized egg yolk-based extender. *Cryobiology,* 71, 329-333, 2015. DOI: 10.1016/j.cryobiol.2015.08.008

24. Gul A, Sahinler N, Onal AG, Hopkins BK, Sheppard WS: Effects of diluents and plasma on honey bee *(Apis mellifera* L.) drone frozen-thawed semen fertility. *Theriogenology*, 101, 109-113, 2017. DOI: 10.1016/j.theriogenology.2017.06.020

25. Cobey SW, Tarpy DR, Woyke J: Standard methods for instrumental insemination of *Apis mellifera* queens. *J Apic Res*, 52, 1-18, 2013. DOI: 10.3896/IBRA.1.52.4.09

26. Maxwell WM, Salamon S: Liquid storage of ram semen: A review. *Reprod Fertil Dev,* 5, 613-638, 1993.

27. El-Kon I: Testing usability of bovine serum albumin (BSA) for preservation of Egyptian Buffalo semen. *Am Eurasian J Agric Environ Sci,* 11, 495-502, 2011.

28. Fang L, Bai C, Chen Y, Dai J, Xiang Y, Ji X, Huang C, Dong Q: Inhibition of ROS production through mitochondria-targeted antioxidant and mitochondrial uncoupling increases post-thaw sperm viability in yellow catfish. *Cryobiology*, 69, 386-393, 2014. DOI: 10.1016/j.cryobiol.2014.09.005

29. Xia J, Ren D: The BSA-induced Ca(2+) influx during sperm capacitation is CATSPER channel-dependent. *Reprod Biol Endocrinol*, 7:119, 2009. DOI: 10.1186/1477-7827-7-119