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Determination of the Osmotic Resistance Changes in Mouse Spermatozoa Using the Classic Osmotic Resistance Test, Short Osmotic Resistance Test and Modified Short Osmotic Resistance Test

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Abstract:

The acrosome is a very important structure on the sperm head. Made up of the outer and inner acrosomal membrane, the acrosome contained enzymes important to digest the zona pellucida of the ovum upon fertilization. Therefore, evaluation of the functional membrane integrity of the acrosome is one of the methods to recognize fertilizing capacity of the sperm. The method commonly used is the osmotic resistance test apart from the hypoosmotic test. Males can be better donors for sperm cryopreservation and this classification can be determine if osmotic resistance test showed excellent results. The traditional or classic osmotic resistance test (ORT) has a longer incubation time of two hours therefore a modified ORT was proposed with an incubation time of 5 minutes referred to as short osmotic resistance test (SORT), and later the modified SORT was introduced where samples were incubated in only 75 mOsmol solution for 5 minutes. As results vary for ejaculated sperm and cauda epididymal sperm, the aim of this study is to detect acrosomal membrane functional integrity in mouse sperm when incubated in classic ORT, short ORT and modified short ORT solutions. Cauda epididymal sperm from twenty mice were collected into Krebs Ringer's Buffer medium and divided into three parts: one for each test. Sperm were pooled, discarded of cell debris and centrifuged on a 2-step discontinuous Ficoll gradient. The sperm was collected from the pellet and incubated for 60 minutes in classic ORT buffer and 5 minutes in short ORT buffer and modified short ORT buffer. Tail swelling, proportion of normal acrosomes (%NAR) and viability was observed from smears taken at intervals of 10 minutes for classic ORT and 1 minute for SORT and modified SORT. Results were subjected to calculation for SORT and modified SORT only which is: $SORT (5 \text{ min}) = 1/2[\%NAR \text{ in } 300\text{mOsmol solution (5 min)} + \% \text{ NAR in } 75\text{mOsmol solution (5 min)}]$ while $\text{modified SORT (5 min)} = 1/2[\%NAR \text{ in samples from sperm morphology} + \% \text{ NAR in } 75\text{mOsmol solution (5 min)}]$. The results showed that classic ORT and modified SORT were found to yield the same findings however classic ORT and SORT showed different findings. The results suggest that the modified SORT can be apply to detect changes in acrosomal integrity to replace classic ORT as it is a rapid test and yields the same results as the classic ORT.

Keywords: Osmotic resistance test (ORT), mice spermatozoa, acrosomal membrane, Short ORT, modified SORT

1. Introduction

One efficient method to recognize fertilization capacity of males is to evaluate the functional membrane integrity of the acrosome (Flesch and Gadella, 2000) especially when the donor semen is stored for artificial insemination. Osmotic resistance test has been shown to be an effective method for the selection of sperm with functional membrane integrity (Kordan et al, 2013) as sperm with a functional plasma membrane will contribute to a successful Intracytoplasmic Sperm Injection (ICSI) technique (Check et al, 2013). Osmosis is defined as the diffusion of water across a semipermeable membrane from a high to low potential until it reaches zero potential (Stillwell, 2016). Osmotic resistance test (ORT) is a test aimed to determine the osmotic tolerance level of a sperm to evaluate the structural and functional membrane integrity especially on cry preserved cells (Agca et al, 2005). ORT allows the determination of the sensitivity of the sperm acrosomal membrane to osmotic pressure changes which can be used as one of the parameters to assess the quality of sperm (Jacyno, et. al., 2013). Fresh sperm incubated under different osmotic pressures reduces the percentage of sperm with a normal acrosome therefore the ORT grades the spermatozoa depending on the functional state of the cell membrane. The functional integrity of the sperm plasma membrane is one of the qualities to assess sperm fertilizing ability by determining the sensitivity of the plasma membrane to osmotic pressure changes (Euginia et al., 2013). Tail functional membrane integrity is determined by the ability of the sperm tail to swell as it allows the osmotic process across the plasma membrane to occur (Ramu and Jayendran, 2012). In ORT, spermatozoa are subjected to moderate hypotonic solution causing the sperm to

undergo initial swelling due to the entry of external water followed by the decrease volume to the initial value in the Regulatory Volume Decrease (RVD) process (Petrunkina et al., 2007a). RVD serves as a function to compensate swelling by controlling osmolytes during hypotonic stress in cell swelling (Guizourn et al., 2000; Zubair et al., 2015). When the hypotonic stress overwhelmed the RVD value, swelling of the spermatozoa induces coiling of the flagella (Chen et al., 2011) and acrosomal swelling. Basically, ORT serves to challenge the sperm with hypoosmotic medium equal to or below than 150mOsm/kg and medium with same osmolality as seminal plasma is approximately 300mOsm/kg for checking the capacity of sperm towards hypoosmotic shock (Sancho and Vilagran, 2013). In a study reported by Kito et al. (2008), BALB/c mice sperm have optimal osmolarity condition of 305mOsmol for effective penetration of the zona pellucida and form male pro-nuclei. The effects can be observed on the acrosome membrane changes whereby the sperm that undergoes osmotic tolerance were presented with altered acrosomal membrane described as swelling and blurry acrosome head observation (Sancho and Vilagran, 2013). ORT is also a useful test to detect functional membrane integrity which is depicted by tail coiling as in HOST. The extent of the viability of sperm incubated in ORT solutions is also important as after maximum tail coiling has occurred, sperm will start to die (Jeyendran et al 2009). Thus, this explains both tail coiling and acrosomal swelling as one of the parameters observed to determine functional membrane integrity of spermatozoa in ORT. Nascimento et al, (2001) introduced three approaches of ORT to test the functional membrane integrity of the sperm. These approaches were the classic ORT, short ORT (SORT) and modified SORT. The classic ORT is the traditional osmotic resistance test (ORT) that have a longer incubation time of one hour while SORT have an incubation time of 5 minutes and the modified SORT is a shorter version of SORT by eliminating one step in the SORT procedure. Nascimento et al (2001) uses boar sperm to test these three approaches where modified SORT was found to yield promising results. The shape of the sperm head in the boar is paddle shape as compared to mouse sperm which has a falciform head shape. Therefore, it would be interesting to observe whether the results would be similar if the same test solutions were applied to cauda epididymal mouse sperm. Cauda epididymal sperm has mature anatomy and physiology similar to ejaculated sperm with the difference of added seminal plasma before being directly released during ejaculation (Falcone & Hurd, 2007). As there is very little data reported on the optimum time for maximum responsive sperm tail in ORT and comparison between classic ORT, short ORT and modified short ORT to identify the best method in determining acrosomal functional membrane integrity of mouse spermatozoa, this study is relevant to be conducted to provide information or baseline values. We report here a study to detect functional integrity of the acrosomal membrane, the optimum time taken for maximum responsive sperm tail coiling and sperm viability of mouse cauda epididymal sperm incubated in classic ORT, short ORT and modified short ORT solutions.

2. Materials and Method

Sexually matured male BALB/C mice were selected to perform this experiment. All animals are provided with food and water compulsory within their need. The rules and regulations of animal ethics from Institute of Animal Care and Use Committee (IACUC) were strictly adhered to conduct animal experiments in Universiti Putra Malaysia (UPM).

2.1. Medium for Sperm Preparation

All the classic Osmotic Resistant Test (ORT), Short Osmotic Resistant Test (SORT) and modified Short Osmotic Resistant Test (modified SORT) medium were prepared prior to the steps of sperm suspension preparation. The three solutions were also maintained at 37°C in water bath before any incubation to prevent cold shock to sperm since the solution were stored in refrigerator below room temperature. Cold or temperature shock is described as the sudden drop of temperature after post-thawing which results in permanent injury to the sperm (Nur et al, 2005). In classic ORT, the medium used is the hypotonic solution of 100mOsmol sodium citrate solution in one-hour incubation time. In SORT, the medium used were the hypotonic solution of 150mOsmol and 75mOsmol sodium citrate solution with concentration of 1.5% and 0.75% respectively whereas in modified SORT, only 75mOsmol sodium citrate solution was used (Nascimento et al., 2001). The 10% form alinsaline solution was also used in both SORT and modified SORT tests (Nascimento et al, 2001). It has been postulated that formaldehyde presence is able to exert a preservation of sperm in longer storage (El-Kon et al., 2009). The 300 mOsmol sodium citrate solution serves as positive control in this experiment.

2.1.1. Spermatozoa Acquisition

Mice were euthanized with chloroform overdose. The cauda epididymides were incised and placed in Krebs Ringers Bicarbonate (KRB) buffer before being minced into small pieces in order to allow sperm flow into the medium. The sperm suspension from the KRB medium was observed under the microscope to ensure the existence of sperm inside the medium prior to incubation with the test solutions.

2.1.2. Selection of Motile Sperm Using Ficoll-Paque PLUS Density Gradient

Selection of motile sperm was by layering on a 2-step discontinuous Ficoll Paque PLUS (GE Healthcare Co., USA) Density Gradient centrifugation of 90% (v/v) and 45% (v/v) density Ficoll layer (Highland et al., 2016). The discontinuous Ficoll gradient separation was described by Haldar et al. (1990) to be effective in yielding high purity and improved intactness of the spermatozoa. This method of motile sperm isolation applies the centrifugation of the sperm suspension to separate the spermatozoa based on their density. The KRB medium was added to the Ficoll solution to produced different density of solutions respectively as described by Haldar et al (1990). The solution of Ficoll was also pre-warmed in 37°C in water bath before layering. The steps continued by layering 2 ml of the sperm suspension onto the top layer gently and carefully (Samardžija et al, 2006). Then, the tube was centrifuged at 300g (Haldar et al., 1990) for 15

minutes. After centrifugation, the viable sperm will be settled down as a pellet at the bottom of the tube. The upper layer was carefully discarded leaving the pellet.

2.1.3. ORT, SORT and Modified SORT Solutions

For classic ORT test, 100mOsmol sodium-citrate solution was added to the sperm suspension following incubation of 60 minutes with time interval of every 10 minutes. Slides were prepared at every interval in triplicates. The isosmotic solution (300mOsm) serves as control for classic ORT. For SORT test, the sperm suspension pellet was incubated with 300 mOsmol sodium-citrate solution followed by the addition of 50 μ l 10% formalin saline performed in water bath at 37°C (Nascimento et al., 2001). Approximately 3 μ l of incubated test solution incubated was pipetted for slides preparation right after 5 minutes incubation has completed. The sperm suspension was then incubated in 75 mOsmol sodium-citrate solution for 5 minutes before smearing onto slides (Nascimento et al., 2001). As for modified SORT, the incubation time is only 5 minutes in 37°C water bath using 75 mOsmol sodium citrate with the sperm suspension following addition of 10% formalin saline (Nascimento et al., 2001). The incubation in 300 mOsmol sodium-citrate solution was omitted to shorten the steps which therefore represent the modification of SORT test.

2.1.4. Staining for Viability

All slides were stained with Eosin Nigrosin for observation of viability. The purpose of staining with Eosin Nigrosin is to differentiate between dead and viable sperm. Nigrosin increases the contrast between the background and sperm heads which provide ease to visualize sperm under light microscope while Eosin serves the function to stain only dead sperm. Dead sperm stained as dark purple while live sperm appear colourless (Agarwal, 2016).

2.1.5. Viewing of Sperm Motility and Morphology

Approximately 100 spermatozoa for each time interval were observed at 400x magnification under compound microscope (B-350 Series, Optika Co., Italy). Interpretation of tail swelling morphology was based on Jeyendran, et. al., (1984) while any indication of acrosomal swelling and blurry outlines were counted as positive according to the observation reported by Nascimento et al ((2001) and Sancho and Vilagran (2013). Non-viable sperm were presented as stained spermas dead cells lose their membrane selective permeability and are permeable to stain while viable sperm remain clear with distinct lines observed on the head and tail of the sperm. This is because the viable sperm have the ability to retain their selective semi permeability of their cell membrane which explains why they do not take up the stain (Highland et al, 2016).

2.1.6. Statistical Analysis

Calculations for SORT and modified SORT (Nascimento et al, 2001) are as below:

$SORT = 1/2 (\% \text{ NAR in washed sperm pellet} + \% \text{ NAR in 75mOsm solution})$

Modified SORT test = $1/2 (\% \text{ NAR in semen pellet} + \% \text{ NAR in 75mOsm solution})$

All data for each group were analyzed statistically by using IBM® SPSS® Statistics software (version 22 for Windows). Data were expressed as means \pm standard error (%). Skewness and Kurtosis of variables were tested for normality distribution, t-test were used to compare between test and control. Statistical significance was set at $p < 0.05$.

3. Results and Discussions

Normal acrosome is acrosome with intact and distinct plasma membrane lines. In comparison to the normal acrosome, the abnormal acrosome is depicted by blurry and swelling area around the head. In this section, only acrosomal changes were assessed to determine the effects of osmolality to the acrosomal membrane. Any tail representation was also recorded where the normal tail is represented by a straight tail of spermatozoa while the abnormal tail is represented by any tail bending or curling according to Jeyenderan et al (1984). For all test solutions, approximately 100 sperm were counted for evidence of acrosomal and tail changes.

3.1.1. Percentage of Normal Acrosomes in SORT and Modified SORT

The percentage of normal acrosomes are compared between SORT and modified SORT only as their incubation time is similar and samples were taken at one-minute intervals. The iso-osmotic solution was used as a control. The calculations for both SORT and modified SORT are shown in Table 1 and Table 2 respectively.

Criteria	Percentage
Number of abnormal acrosomes found in washed sperm pellet.	2
Percentage morphology of normal acrosome (NAR)	98
Number of abnormal acrosomes found in 75mOsm solution	7
Percentage morphology of normal acrosome (NAR)	93
SORT = $1/2 (\% \text{ NAR in washed sperm pellet} + \% \text{ NAR in 75mOsm solution})$	95.5

Table 1: Calculations for SORT

Criteria	Percentage
Number of abnormal acrosomes found in iso-osmotic solution	3
Percentage morphology of normal acrosome (NAR)	97
Number of abnormal acrosomes found in 75mOsm solution	7
Percentage morphology of normal acrosome (NAR)	93
Modified SORT test = 1/2 (% NAR in semen pellet + % NAR in 75mOsm solution)	95

Table 2: Calculations for Modified SORT

Figure 1 presents the comparison of normal acrosome percentage after incubation of 5 minutes in control, SORT and modified SORT respectively. The control medium (300mOsm) has the highest number of percentage of normal acrosome which is 97% compared to SORT and modified SORT percentage of normal acrosomes which is 95.5% and 95% respectively.

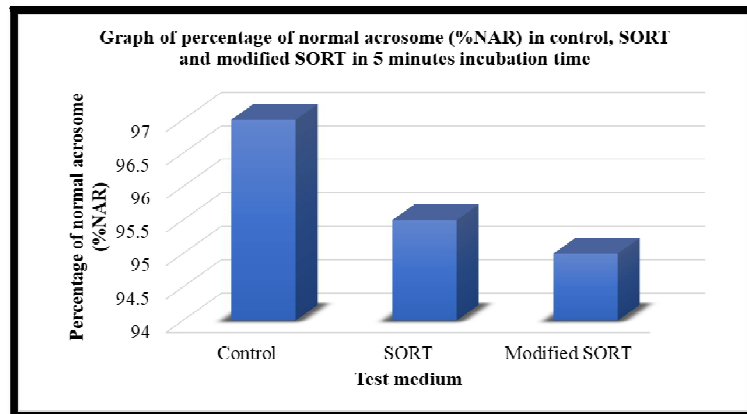


Figure 1: Percentage of Normal Acrosomes in ISO-Osmotic Solution (Control), SORT and Modified SORT

3.1.2. Number of Normal Acrosomes in Classic ORT Solution as Compared to ISO-Osmotic Solution

The number of normal acrosomes in Classic ORT solution is compared with iso-osmotic solution which serves as the control. The Classic ORT is not compared with the SORT and Modified SORT as the incubation time for the latter is only 5 minutes. Figure 2 shows the overall view of Classic ORT of 100mOsm with lowered percentage of normal acrosomes as to compared to control solution of 300mOsm. Based on the graph, it is observed that the percentage of normal acrosomes in Classic ORT and control is not exceeding 10% and 3% respectively.

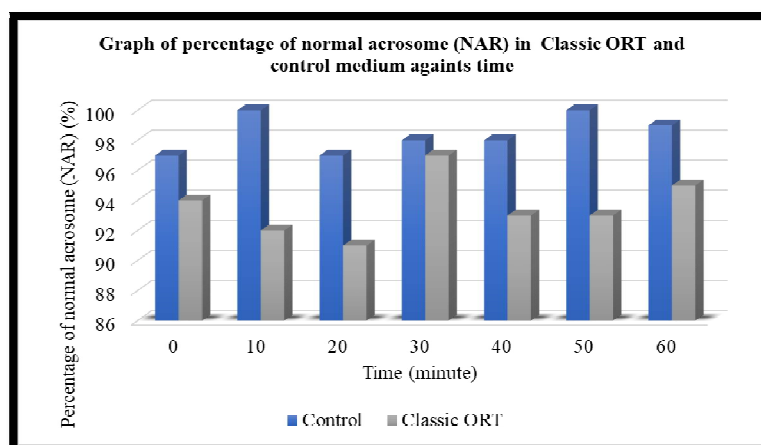


Figure 2: Normal Acrosome Percentage in Classic ORT and ISO-Osmotic Solution against Time

3.1.3. Morphology of Tail Swelling in Test Medium (Classic ORT)

In this section, tail coiling was determined by the representation of flagellum coiling starting from the distal end of the tail region towards the mid-piece described by Jeyendran et al. (1984) in their study of functional membrane integrity of spermatozoa. This representation was further supported by the evidence in unpublished data on the tail appearance that starts coiling from the end-piece towards the upper part of flagellum. Any other tail bending was excluded including any tail angulation that occur at the cytoplasmic droplets located on a point between the middle piece and principle piece. Figure 3 depicts the tail coiling in Classic ORT is far higher than in control medium.

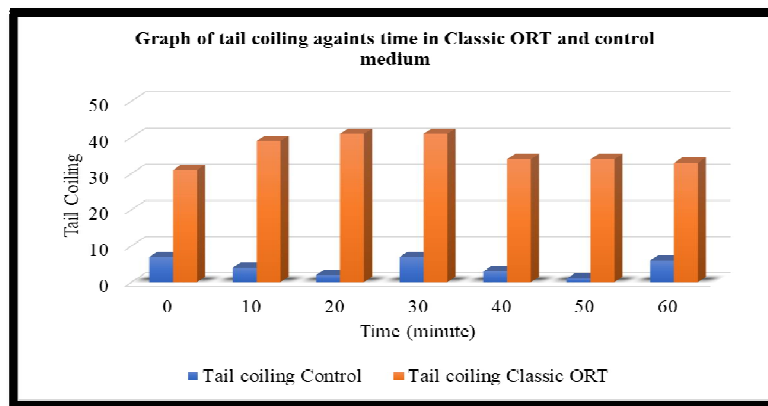


Figure 3: Percentage of Tail Coiling in Classic ORT and Control Medium against Time

3.1.4. Evaluation and Interpretation of Non-Viable and Viable Spermatozoa

Assessing viability of spermatozoa is unstained spermatozoa falls into the live category while stained spermatozoa falls into the dead category. Dead normal spermatozoa were characterized with stained and straight tail with normal acrosome while live normal spermatozoa were characterized with clear white representation that distinguished it from the background colour. There are also live spermatozoa but with tail curling and dead spermatozoa with normal tails. Figure 4 and 5 depicts the live and dead spermatozoa with normal or abnormal tails (curling) in iso-osmotic (control) solutions and Classic ORT solutions respectively.

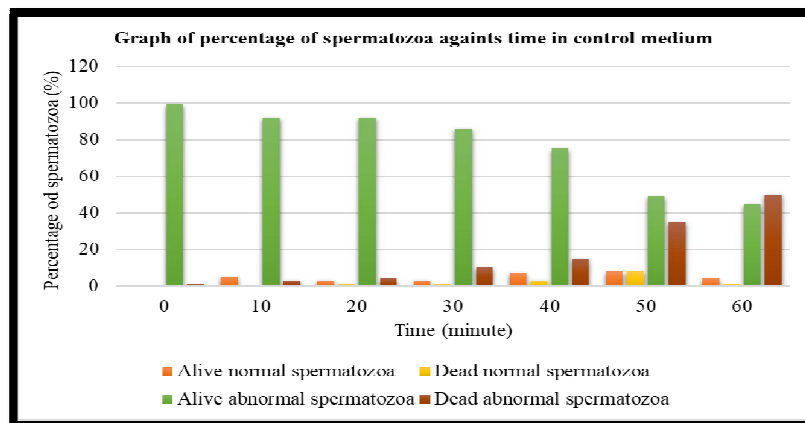


Figure 4: Percentage of Viable and Non-Viable Spermatozoa with Normal or Tail Curling in ISO-Osmotic Medium

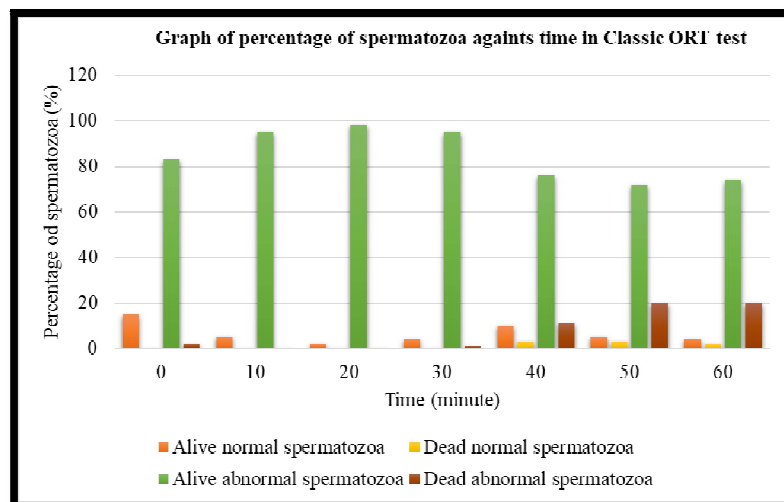


Figure 5: Percentage of Viable and Non-Viable Spermatozoa with Normal or Tail Curling in Classic ORT Medium

4. Discussions

The results on percentage of normal acrosomes (%NAR) using the independent test T yielded a significant value of p=0.0001 between classic ORT and control medium. Therefore, classic ORT is significantly different from the control in

percentage of normal acrosomes. In contrast, the one sample T test was conducted between the means of Classic ORT and SORT yielded non-significant results ($p = 0.043$). In SORT, the incubation in 300mOsm was applied while in modified SORT, this step was eliminated and replaced by assessing the sample of washed sperm pellet directly under the microscope. Based on the percentage of normal acrosomes in 300mOsm, the results yielded not more than 3% abnormal acrosomes. However, in modified SORT, the incubation in 300mOsm is replaced with direct observation on washed sperm samples rendering the result to be unaffected. In classic ORT, the results on the percentage of normal acrosome indicated that there are not more than 10% abnormal acrosomes present. Both Classic ORT and control yielded very low percentage of acrosomes affected by the test solutions. This is because the acrosome membrane needs to be tough and maintained the enzymes capsulated before they can reach the oocyte because if the sperm lose their acrosomes before encountering the oocyte, they are unable to bind to the zona pellucida and thereby results in failure in fertilization (Bowen, 2000). This shows the importance of acrosomal integrity assessment in semen analysis (Bowen, 2000). As for the normal acrosome percentage of spermatozoa, the incubation of 5 minutes in control, SORT and modified SORT respectively showed that the control medium (300mOsm) has the highest number of percentage of normal acrosomes as compared to SORT and modified SORT. The optimum time for tail coiling in hypotonic medium of 100mOsm (classic ORT) medium is 20 to 20 minutes in mice sperm as compared to 45 to 60 minutes in rat sperm (Ong and Sabrina, unpublished data). This suggest that the mouse sperm is less resistant and sensitive to changes in osmolality as compared to rat sperm.

As for the comparison of normal acrosomes in control, SORT and modified SORT) with incubation of 5 minutes respectively, the results showed that the control medium (300mOsm) has the highest number of percentage of normal acrosomes (97%) followed by SORT (95.5%) and modified SORT (59%). T test analysis for optimum tail coiling between classic ORT and control provide a significant value of $p = 0.0001$ showing a significant difference between classic ORT and control medium for optimal tail swelling. It is observed that the optimum time for tail coiling in BALB/c mice was between 20 to 30 minutes in classic ORT suggesting that the best osmolality range for optimum tail coiling is 100mOsm medium for mouse sperm. The pattern of viability which is time dependent in both classic ORT and control shows almost similar readings from minute 0 to 40 suggests that the viability of the spermatozoa was not highly affected by the different osmolality of hypoosmotic solutions but rather on prolong incubation and once acrosome reaction has occurred, the sperm will slowly die.

The staining method provide a limitation of setting a valid reliable benchmark as different intensity of color takes up by the spermatozoa provide different interpretation from different views. This is because the plane of the spermatozoa being falciform differs upon smearing technique causing different interpretations of staining uptake. The confusion as well applies on the tail curling appearance. Therefore, only tail appearance that fits the description findings in Jeyendran et al. (1984) were evaluated. This provide a much more convenient way to really identify and classify the spermatozoa in accordance to the test applied. The evidence of spermatozoa clumping also affect the overall reading as the tail morphology cannot be evaluated personally based the manner where the sperm intertwine with each other. This phenomenon was explained by Firman and Simmons (2009) that the event was due to the nature of mice sperm's hook to form sperm 'trains' beneficial in a competitive situation.

From the results obtained for this preliminary study, we suggest that the modified SORT can be used to replace Classic ORT at least for mice spermatozoa as they both yielded similar results.

5. Conclusions

In conclusion, the classic ORT and modified SORT were found to yield the same finding for the percentage of normal acrosome but showed different findings for percentage of normal acrosome between classic ORT and SORT. This suggests that the modified SORT can be used to detect changes in acrosomal integrity to replace classic ORT as it is a rapid test and yields the same results as the classic ORT.

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