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Sperm Membrane Functionality in the Dog Assessed by Flow Cytometry

C Cheuquemán[1], P Bravo[1], F Treulén[1], LC Giojalas[2], J Villegas[1,2,3], R Sánchez[1,2,3,4] and J Risopatrón[1,2,3,4,5]

[1]*Centre of Reproductive Biotechnology (BIOREN-CEBIOR), Faculty of Medicine, University of La Frontera, Temuco, Chile;* [2]*Centre for Cellular and Molecular Biology, National University of Cordoba, Cordoba, Argentina;* [3]*Department of Internal Medicine;* [4]*Department of Preclinical Sciences;* [5]*Department of Basic Sciences, Faculty of Medicine, University of La Frontera, Temuco, Chile*

Contents

The objective assessment of sperm function increases the chances of predicting the fertilizing capacity of a fresh semen sample or diagnosing infertility problems. In this study, the available flow cytometry technique was used to determine the membrane functional capacity of canine spermatozoa. The second fractions of ejaculates from six dogs were pooled, and samples (n = 26) processed to determine the variables: sperm viability and plasma membrane integrity by Sybr-14/Pi staining; phosphatidylserine (PS) translocation by Annexin-V-FITC/PI labelling; acrosome membrane integrity by FITC-conjugated *Pisum sativum agglutinin*/PI labelling; and mitochondrial membrane potential ($\Delta\Psi$m) by staining with JC-1. Means for the 26 examined samples indicated that 82.66 ± 2.8% of the viable spermatozoa showed an intact plasma membrane, 8.4 ± 2.6% were moribund, 72.7 ± 16% had an intact acrosome, 80.9 ± 17% had high $\Delta\Psi$m and 8.1 ± 11% had PS translocation with a PS translocation index of 2.1 ± 3%. Motility was only correlated with PS translocation (R = 0.3901; p = 0.0488), and acrosome membrane integrity was correlated with PS translocation (R = −0.5816; p = 0.0018). This study provides objective physiological data on the functional capacity of canine spermatozoa.

Introduction

The purpose of evaluating semen, especially fresh semen, is to predict its fertilizing capacity and check the normal functioning of the testicles and epididymis in an individual male for breeding or assisted reproduction techniques (Peña 2004).

Conventional sperm analysis techniques based on light microscopy are gradually being replaced by fluorescence microscopy or flow cytometry procedures requiring fluorescent staining techniques (Peña et al. 2001). Through flow cytometry, multiple sperm characteristics can be simultaneously assessed, and this approach seems promising for evaluating sperm quality and understanding sperm functionality (Petrunkina et al. 2007).

The benefits of flow cytometry procedures are that they are highly quantitative, repeatable and sensitive (Christensen et al. 2004, 2005) and the structural characteristics of a large number of spermatozoa can be determined in a short period of time (Rijsselaere et al. 2005). Effectively 10,000 cells can be analysed in under a minute. Thus, large data sets can be generated providing the statistical validity needed for correlations to be made between quality variables and the fertilizing capacity of spermatozoa (Silva and Gadella 2006). To fertilize an oocyte, spermatozoa need to have many attributes such that a test that evaluates a single variable will not be able to detect single defective cells, and this may lead to overestimation of the number of spermatozoa with good fertilizing capacity in a semen sample (Graham and Mocé 2005).

Flow cytometry can also be used as a diagnostic tool to predict the fertility of a given male, specially the cells membrane (Gillan et al. 2005). Plasma membrane integrity is essential for a spermatozoon's fertilizing capacity (Rijsselaere et al. 2005). If the sperm plasma membrane is not functionally intact, the sperm is defined as deteriorated (dead), which means it is not capable of fertilizing an oocyte *in vivo* (Graham and Mocé 2005). Therefore, its integrity must be necessarily tested (Silva and Gadella 2006). Sybr-14 plus propidium iodide (PI) staining is one of the most commonly used methods to determine the viability and integrity of the plasma membrane (Garner and Johnson 1995). Early acrosome reactions render sperm infertile, and therefore, acrosome membrane integrity needs to be assessed before an assisted reproduction procedure (Silva and Gadella 2006). Acrosome membrane integrity is commonly determined using fluorescent-conjugated lectins, such as *Pisum sativum agglutinin*, conjugated to fluorochromes such as FITC-PSA (Kawakami et al. 2002). PSA binds specifically to the acrosome content by interaction with the proacrosin, which is a glycoprotein with saccharide groups (Peña et al. 1999).

Another important indicator of sperm functionality that can be assessed using specific fluorescent markers is the mitochondrial membrane potential ($\Delta\Psi$m). It is an indicator of sperm functionality that can be assessed using specific fluorescent markers (Volpe et al. 2009). Mitochondrial membrane function can be estimated through $\Delta\Psi$m using the fluorescent probe, 5,5′,6,6′-tetrachloro-1,1′, 3,3-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), which is able to discriminate between high and low $\Delta\Psi$m (Smiley et al. 1991). Also, Phosphatidylserine (PS) translocation is an indicator of asymmetry alterations of plasma membrane associated with the mechanism of apoptosis in somatic cells although in human spermatozoa the PS translocation has been demonstrated to occur during the process of capacitation independently of apoptosis (De Vries et al. 2003; Martin et al. 2005). PS translocation can be identified by Annexin-V, a calcium-dependent PS-binding protein, which when conjugated to a fluorochrome such as FITC permits the identification of cells with exposed PS (Silva and Gadella 2006).

The aim of this study was to assess the functional capacity of the sperm plasma membranes by different methods applying flow cytometry to provide standardized physiological data on canine spermatozoa.

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Materials and Methods

This study was approved by the corresponding institutional review boards. Unless otherwise indicated, all chemicals used were purchased from Sigma (St Louis, MO, USA). All solutions were prepared using water from a Milli-Q Synthesis System (Millipore, Bedford, MA, USA).

Animals

Sperm samples were obtained from six clinically healthy dogs (two German Shepherds, two Golden Retriever and two Labradors) of weight 20–25 kg and age 3–5 years, belonging to the Biotherium Centre of Reproductive Biotechnology (CEBIOR), Faculty of Medicine, University of La Frontera, Temuco, Chile. The dogs were individually housed and kept on a balanced diet with free access to water and exercised daily throughout the experiment. The animals' health state was periodically checked by a veterinarian. The dogs were routinely used as semen donors and had been trained for semen collection before the study outset.

Semen collection and processing

In each experimental trial (n = 26), semen was collected once a week by manual manipulation into a pre-warmed graduated test tube, and only the second fraction of each ejaculate was used (Linde-Forsberg 1995; Kutzler 2005). After collection, motility, concentration and morphology were determined in aliquots of each ejaculate to ensure adequate semen quality. The percentage of motile sperm was estimated subjectively by observation under a phase contrast microscope (Carl Zeiss, Jena, Germany) on a heated stage at 37°C at a magnification of 400×. To assess motility, 10 μl of semen was placed on a slide and covered with a coverslip. Percentages of motile and progressively motile spermatozoa were determined in a minimum number of 300 sperm in at least six different microscopy fields. The mean of these six successive estimations was recorded as the final motility score (Risopatrón et al. 2002; Kim et al. 2010). Sperm concentration was determined using a Neubauer counting chamber. The percentages of viable spermatozoa with a normal morphology were assessed by double staining (Didion et al. 1989; Risopatrón et al. 2002) before observation under a light microscope. Only ejaculates showing sperm concentrations $\geq 200 \times 10^6$ spermatozoa/ml, progressive motility ≥70% and normal morphology ≥80% were included in the study. These sperm variables are generally considered to indicate good sperm quality and could thus be correlated with the integrity of the plasma, acrosome and mitochondrial membranes. Aliquots of samples showing similar spermatozoa concentrations were pooled to increase volume and eliminate variability among the different samples (Verstegen et al. 2005).

The semen pool was centrifuged at $720 \times g$ for 3 min (Rijsselaere et al. 2002), the supernatant was discarded, and the pellet was washed twice by centrifugation at $720 \times g$ for 3 min in TRIS buffer (Tris buffer containing glucose; Peña et al. 2003). The suspension was adjusted to 3×10^6 cells/ml in TRIS buffer. Subsequently, aliquots for the different sperm evaluation techniques were prepared from this pool.

Viability and plasma membrane integrity

Viable spermatozoa with intact plasma membranes were detected using SYBR-14/PI (LIVE/DEAD® Sperm Viability kit; Molecular Probes cat no L-7011, Eugene, OR, USA) according to the manufacturer's instructions with some modifications. A volume of 1.25 μl of Sybr-14 (100 nM) was added to 250 μl of sperm suspension (3×10^6 cells/ml). After 5 min of incubation at 37°C, 1.25 μl PI (12 μM) was added and the suspension incubated for a further 5 min at 37°C. The stained sperm sample was washed with 1 ml of TRIS buffer by centrifuging at $300 \times g$ for 5 min. The supernatant was discarded, and the cells were resuspended in 400 μl of TRIS buffer and immediately analysed by flow cytometry. Spermatozoa were classified as (see Fig. 1a): viable with an intact plasma membrane (PI negative/Sybr-14 positive/), dead (PI positive/Sybr-14 negative), or moribund (PI positive/Sybr-14 positive). The results of each trial were analysed three times.

Phosphatidylserine (PS) translocation

To determine PS translocation in the sperm membrane, the Annexin-V-FITC/PI apoptosis detection kit (APOPTEST™-FITC; Nexins Research, Hoeven, The Nether-

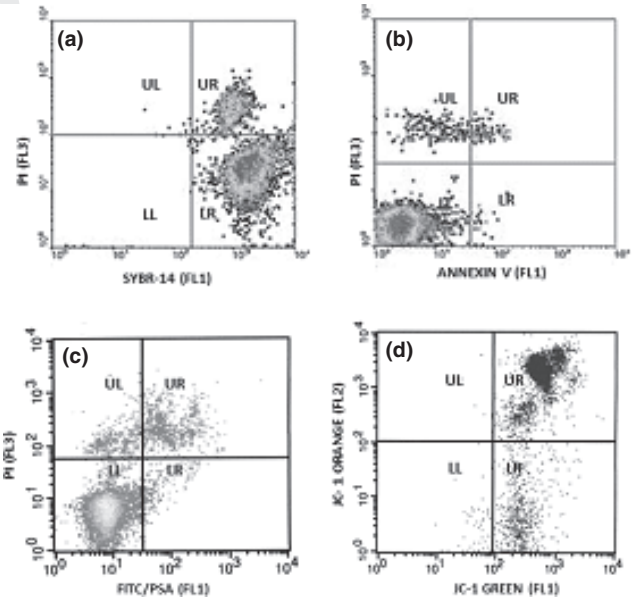

Fig. 1. Flow cytometry analysis of a sperm population sample. (a) Sybr-14/PI fluorescent staining. UR: moribund spermatozoa, LR: viable spermatozoa with an intact plasma membrane. UL: dead spermatozoa. (b) Annexin-V-FITC/PI fluorescent staining. UR: dead spermatozoa showing PS translocation. LR: viable spermatozoa showing PS translocation. UL: dead spermatozoa with no signs of PS translocation. LL: viable spermatozoa with no signs of PS translocation. (c) FITC-PSA/PI fluorescent staining. UR: dead spermatozoa with a damaged acrosome membrane. LR: viable spermatozoa with a damaged acrosome membrane. UL: dead spermatozoa with an intact acrosome membrane. LL: viable spermatozoa with an intact acrosome membrane. (d) JC-1 fluorescent staining. UR: spermatozoa with high ΔΨm. LR: spermatozoa with low ΔΨm

LOW RESOLUTION FIG

lands) was used following the manufacturer's instructions with modifications. A volume of 250 μl of sperm suspension (3×10^6 cell/ml) was centrifuged at $300 \times g$ for 5 min. The supernatant was discarded, and the pellet of spermatozoa was resuspended in 96 μl of the Annexin-V binding buffer (1 ×) provided in the kit. After adding 1 μl of Annexin-V-FITC (25 μg/ml) and 2.5 μl of PI (250 μg/ml) stock solution, the sample was incubated for 10 min at 4°C in the dark. After incubation, the sperm suspension was washed by centrifuging at $300 \times g$ for 5 min (Kim et al. 2010), the supernatant was discarded, and the pellet was resuspended in 300 μl of binding buffer and immediately analysed by flow cytometry. The spermatozoa were classified as (see Fig. 1b): viable without PS translocation (PI negative/AN negative); viable with translocated PS (PI negative/AN positive); dead (PI positive/AN positive or PI positive/AN negative) (Januskauskas et al. 2003; Chaveiro et al. 2007). The results of each trial were analysed three times.

Acrosome membrane integrity

Acrosome membrane integrity was assessed by fluorescent staining with FITC-conjugated *Pisum sativum agglutinin* (FITC-PSA)/PI (kit FITC-PSA/PI; St Louis, MO, USA) (Peña et al. 1999). To this end, 2.5 μl of FITC-PSA/PI (2 μM in saline phosphate buffer) was added to a volume of 250 μl of sperm suspension (3×10^6 spermatozoa/ml) followed by incubation for 15 min at 38°C in the dark. After centrifuging at $300 \times g$ for 5 min, the supernatant was discarded, and the pellet of spermatozoa was resuspended in 400 μl of TRIS buffer and immediately analysed by flow cytometry. The spermatozoa were classified as (see Fig. 1c): viable with an intact acrosome membrane (PI negative/FITC-PSA negative) or viable with a damage acrosome membrane (PI negative/FITC-PSA positive-), dead with an intact acrosome membrane (PI positive/FITC-PSAnegative) or dead with a damaged acrosome membrane (FITC-PSA + /PI +). The results of each trial were analysed three times.

Mitochondrial membrane potential (ΔΨm)

To evaluate the ΔΨm of the spermatozoa, 5,5′,6,6′–tetrachloro-1,1′,3,3′ tetraethylbenzymidazolylcarbocyanine iodine known as JC-1 was used. This test was performed using the Mitochondrial Permeability Detection kit AK-116 (MiT-E-Ψ™; BIOMOL International LP, Plymouth Meeting, PA, USA) following the manufacturer's instructions. A volume of 250 μl of sperm suspension (3×10^6 cells/ml) was centrifuged at $300 \times g$ for 5 min, and the supernatant was discarded. The resultant pellet of spermatozoa was resuspended in 250 μl of JC-1 working solution (3 mM JC-1 in TRIS buffer) and incubated for 15 min at 38°C in the dark. Next, the cell suspension was centrifuged at $300 \times g$ for 5 min, the supernatant was discarded, and the sperm pellet was resuspended in 400 μl TRIS buffer and immediately analysed by flow cytometry (Fig. 1d). The spermatozoa with high ΔΨm were the cell population with high orange fluorescence (FL3 > 10^2, see Fig. 1d), and spermatozoa with low ΔΨm were the cell population with orange fluorescence < 10^2 (FL3) (Martinez-Pastor et al. 2004). The analysis of each trial was replicated three times.

Flow cytometry

Fluorescence was detected in a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a standard optic. The cells were excited at a wavelength of 488 nm using an argon laser. A minimum of 10 000 spermatozoa were included in each analysis. To define specifically the settings of canine sperm population, forward-Scatter-Height (FSC-H) was plotted against sScatter-height (SSC-H). The data were provided on a logarithmic scale and analysed using CellQuest Pro Software (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

Data were analysed using GraphPad Prism® software, version 5.0 (GraphPad Software, San Diego, CA, USA). For the different functional parameters evaluated by flow cytometry, the data are provided as average percentages ± standard deviation (SD) recorded for the 26 samples. Spearman's correlation for nonparametric data was used to detect correlations among the sperm variables examined. The level of significance was set at $p < 0.05$.

Results

Through flow cytometry, we were able to determine the most important variables associated with the functions of the different membranes (plasma, acrosome and mitochondrial) present in sperm (Fig. 1).

The mean rates of motility and progressive motility recorded were 92.2 ± 2.3% and 87.2 ± 5.0%, respectively. The percentage of viable spermatozoa with intact plasma membrane was 82.66 ± 2.8% and 8.4 ± 2.6% of the viable spermatozoa having a damaged plasma membrane were classified as moribund.

The percentage of viable spermatozoa that showed PS translocation was 1.8 ± 2.6%. The PS translocation index, calculated as the ratio between the percentage of live spermatozoa showing PS translocation and the total percentage of live spermatozoa, was 2.1 ± 3%.

The percentage of viable spermatozoa with an intact acrosome membrane was 72.7 ± 16%, while a damaged acrosome was only detected in 5 ± 9%. High ΔΨm was detected in 80.9 ± 17% of the sperm population examined.

Finally, motility was correlated with PS translocation ($R = 0.3901$; $p = 0.0488$), and acrosome membrane integrity was correlated with PS translocation ($R = -0.5816$; $p = 0.0018$). No significant correlations were detected among the remaining sperm variables examined (Table 1).

Discussion

The quality or fertilizing capacity of spermatozoa, although reflected by their motility, viability and normal

Table 1. Probability of correlation between the sperm parameters studied

Functional parameters	p-value	r	Correlation
(a) Motility-membrane integrity	0.4933	0.2571	ns
(b) Motility-acrosome membrane integrity	0.1775	−0.2728	ns
(c) Motility-PS translocation	0.0488	0.3901	Positive correlation
(d) Motility-mitochondrial membrane potential	0.4458	−0.1563	ns
(e) Membrane integrity-acrosome membrane integrity	0.2926	0.2145	ns
(f) Membrane integrity-PS translocation	0.7311	−0.07079	ns
(g) Membrane integrity-mitochondrial membrane potential	0.5164	−0.1333	ns
(h) Acrosome membrane integrity-PS translocation	0.0018	−0.5816	Inverse correlation
(i) Acrosome membrane integrity-mitochondrial membrane potential	0.1746	0.2746	ns
(j) PS translocation-mitochondrial membrane potential	0.8582	0.03683	ns

morphology, is also dependent on their functional and structural competence (Kim et al. 2010). In the present study, newly available technique of flow cytometry was used to assess several characteristics of good-quality dog sperm, complementing the more conventionally variables as motility, viability and normal morphology. These determined variables were comparable to the figures reported by other authors for canine sperm (Peña 2004; Kustritz 2007; Lopes et al. 2009; Kim et al. 2010).

Most current sperm viability tests are based on determining sperm cell plasma membrane integrity, because an intact competent plasma membrane is needed to fertilize an oocyte (Graham and Mocé 2005). In the present study, through Sybr-14/PI fluorescent staining, we were able to detect a high percentage of viable spermatozoa with an intact plasma membrane (82.66 ± 2.8%). In previous reports, rates determined by the HOST test have been higher (proximally 90%) (Lopes et al. 2009) perhaps because PI staining in flow cytometry excludes moribund spermatozoa.

Our overall motility rate (92.2 ± 2.3%) contrasts with the percentage of viable sperm that had an intact plasma membrane (82.66 ± 2.8%) determined by SYBR-14. Plasma membrane integrity and good motility are known to be highly correlated (Schäfer-Somi and Aurich 2007). This discrepancy could be attributed to the subjective nature of our motility test, indicating a reduced number of cells counted under the light microscope. Our progressive motility figure (87.2 ± 5.0%) was, however, more consistent with the plasma membrane integrity result.

The percentage of viable spermatozoa with an intact acrosome detected here was almost double the rates of 38 ± 7% using flow cytometry and 38.4 ± 9.1% with epifluorescence microscopy reported by Peña et al. (1999). These differences could be related to the high number of viable sperm with an intact plasma membrane present in our pooled semen samples. In fact, high viability values have been recently described for canine semen assessed by flow cytometry (Volpe et al. 2009).

Phosphatidylserine translocation is a marker of plasma membrane destabilization (Kim et al. 2010). The PS translocation index recorded here was lower than that reported by Kim et al. (2010) for canine spermatozoa in fresh semen samples. This difference could also be explained by the lower percentage of spermatozoa with an intact plasma membrane detected in the samples examined by these authors.

The ΔΨm observed in the present study was higher (63.9–97.9%) than the range (53–87%) reported by Volpe et al. (2009). This again could be attributed to the fact that our semen samples were obtained from dogs whose sperm quality was good according to the results of conventional tests (Kustritz 2007).

Acrosome membrane integrity was correlated with PS translocation ($R = -0.5816$; $p = 0.0018$; Table 1h). This finding is supported by the fact that once the acrosome reaction has occurred, the spermatozoon plasma membrane rapidly loses its integrity (Peña et al. 1999).

No significant correlation was detected between sperm motility and ΔΨm (Table 1d), contrary to previous findings (Volpe et al. 2009). Notwithstanding, in a recent study, Nascimento et al. (2008) were also unable to observe any correlation between motility and mitochondrial membrane potential, and these authors suggested that the inhibition of mitochondrial ATP production was not sufficient to reduce sperm motility. Mitochondrial membrane potential has been described as one of the most sensitive parameters for evaluating sperm function, and its reduction has been taken to indicate an imminent loss of the sperm's capacity for motility, fertility and survival in the female reproductive tract (Kasai et al. 2002; Grunewald et al. 2008). The loss of ΔΨm is a well-known apoptotic marker in somatic cells, which has been related to an uncoupling of the electron transport chain for ATP synthesis and an increased generation of reactive oxygen species (Rajender et al. 2010).

In conclusion, through flow cytometry, a set of objective data on the functional and structural characteristics of the sperm membranes was obtained in a large number of fresh semen samples containing good-quality sperm, as indicated by routine viability, morphology and motility tests. These results have implications for improving the efficiency of breeding dog selection and also provide direction for future studies designed to evaluate assisted reproduction techniques.

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

All authors similarly contributed to reviewing the literature and writing the text. The work was coordinated by Jennie Risopatrón.

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Author's address (for correspondence): Prof. M.Sc. Jennie G. Risopatrón, Centre of Reproductive Biotechnology (BIORENCEBIOR), Faculty of Medicine, University of La Frontera, Avd. Francisco Salazar 01145, Temuco, Chile.
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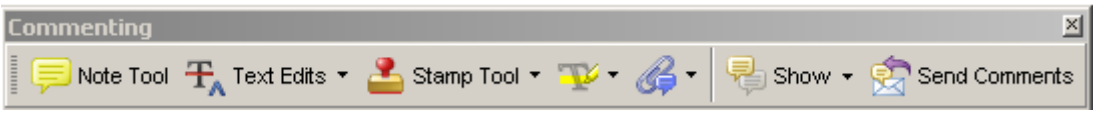
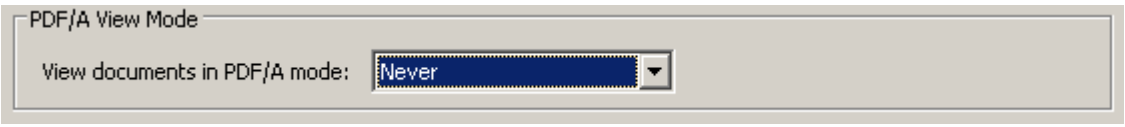


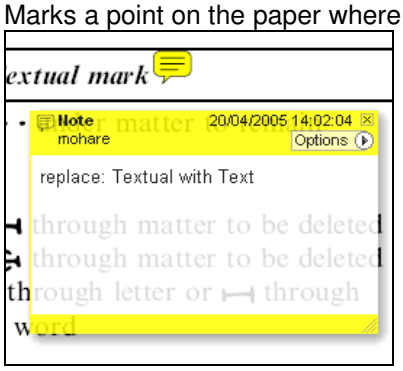
If you experience problems annotating files in Adobe Acrobat Reader 9 then you may need to change a preference setting in order to edit.

In the "Documents" category under "Edit – Preferences", please select the category 'Documents' and change the setting "PDF/A mode:" to "Never".



Note Tool — For making notes at specific points in the text

Marks a point on the paper where a note or question needs to be addressed.

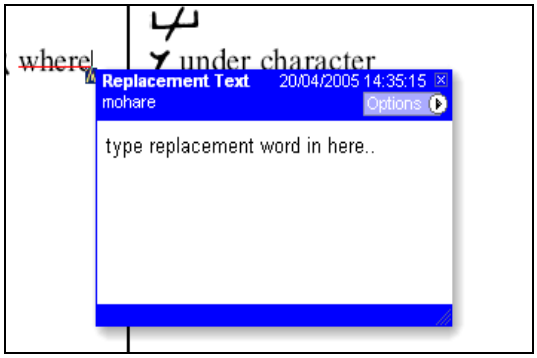


How to use it:

1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.

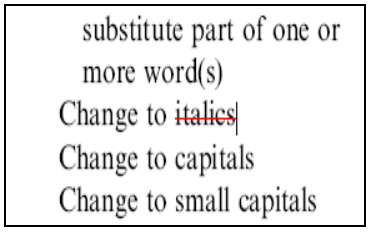


How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
6. Click outside of the blue box to close

Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

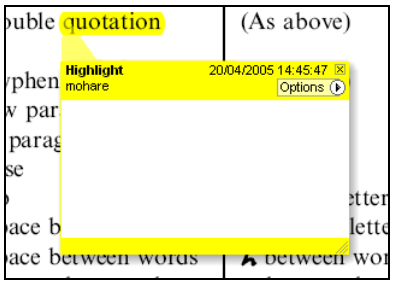
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the 'standard business' selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

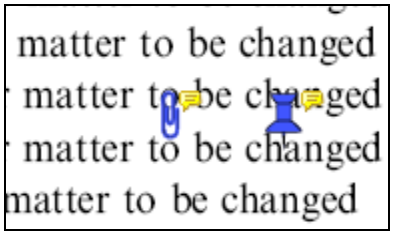


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

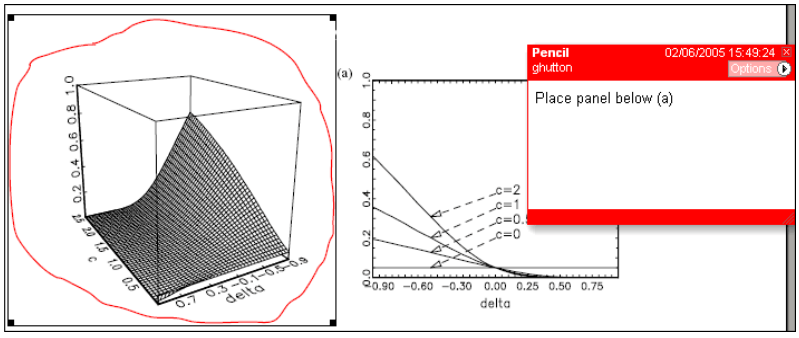


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: