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Research Article

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Investigation● of pituitary adenylate cyclase activating polypeptide in human gynecological and other biological fluids by using MALDI TOF mass spectrometry†

R. Brubel,[a] D. Reglodi,[a] E. Jambor,[b] M. Koppan,[a,c] A. Varnagy,[c] Zs. Biro,[d] P. Kiss,[a] V. Gaal,[d] A. Matkovits,[a] J. Farkas,[a] A. Lubics,[a] J. Bodis,[c] Cs. Bay,[c] B. Veszpremi,[c] A. Tamas,[a] J. Nemeth[e] and Laszlo Mark[b]*

Pituitary adenylate cyclase activating polypeptide (PACAP) is a multifunctional and pleiotropic neuropeptide. PACAP has diverse effects in the endocrine system, among others, it plays important roles in oogenesis, implantation and development of the nervous system. However, it is not known whether PACAP is present in the fluids of the human reproductive organs. The aim of the present study was to determine, by means of mass spectrometry and radioimmunoassay, whether PACAP is present in human amniotic fluid, ovarian follicular fluid and cervico-vaginal fluid. Samples were obtained from healthy adult volunteers. Our MALDI TOF and MALDI TOF/TOF spectrometry results show that PACAP38 is present in all of the follicular fluid samples, and PACAP-like immonoreactivity was also measured by radioimmunoassay. However, we did not find the characteristic peak representing the unmodified 38 amino acid form of the peptide in normal cervico-vaginal smear and amniotic fluid samples. Furthermore, we analyzed other body fluids for comparison, such as human nasal fluid, saliva and aqueous humor. PACAP was not found in these latter samples. In summary, the present study provides evidence for the presence of PACAP in human follicular fluid, suggesting a role in oocyte function, but determination of the exact physiological significance awaits further investigation.

Keywords: cervico-vaginal fluid; amniotic fluid; follicular fluid; nasal fluid; saliva; aqueous humor; mass spectrometry; radioimmunoassay

Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) was originally isolated from the hypothalamus, based on its cAMP-increasing effect in pituitary cells.[1,2] PACAP occurs in two amino acid forms: PACAP38 and PACAP27, with the 38 amino acids form being predominant in human tissues.[1–5] Although the last two decades since its discovery have revealed that PACAP is much more than 'just' a hypothalamo/hypophyseal peptide, its functions played in the endocrine system are still in focus of research.[3–6] Regarding reproductive endocrinology, PACAP has been shown to play a role in the regulation of gonadotropin secretion,[3,7,8] fertility, receptivity, implantation, reproductive behavior[9–11] and placental functions.[12] Furthermore, PACAP delays puberty[13] and reduces follicular apoptosis in the ovary.[14]

Mass spectrometry (MS) is a high-throughput technique to analyze peptide/protein composition of biological fluids.[15] We have previously shown the presence of PACAP in human serum and human breast milk using matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) and matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF) MS.[16] This technique provides a powerful tool for investigating other biological samples and fluids as well. Peptide profiling of biological samples has become increasingly important in search for biomarkers and in investigation of differences between normal and pathological fluids.[17] Given the importance of endogenous PACAP in reproductive physiology, the first aim of the present study was to investigate the occurrence of PACAP in human fluids of the reproductive system.

Ovarian follicular fluid is the product of granulosa and theca cells as well as a plasma filtrate through the wall of the developing follicle.[17] It functions as a culture medium for the developing oocyte. Since PACAP has been shown to play a role in follicular development,[14,18,19] it was of interest to examine whether the peptide occurs in the follicular fluid using MALDI TOF and TOF/TOF MS, and if present, to detect its levels in follicular fluid samples using radioimmunoassay (RIA).

* *Correspondence to: Laszlo Mark, Department of Biochemistry and Medical Chemistry, University of Pecs, Szigeti u 12, 7624 Pecs, Hungary. E-mail: laszlo.mark@aok.pte.hu*

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a *Department of Anatomy, University of Pecs, Pecs, Hungary*

b *Department of Biochemistry and Medical Chemistry, University of Pecs, Pecs, Hungary*

c *Department of Obstetrics and Gynaecology, University of Pecs, Pecs, Hungary*

d *Department of Ophthalmology, University of Pecs, Pecs, Hungary*

e *Department of Pharmacology and Pharmacotherapy, University of Debrecen, Debrecen, Hungary*

Amniotic fluid is partially a plasma filtrate and a product of the fetal epithelial cells. Its integrity is essential for the normal development of the fetus.[15,19] The diagnostic value of amniotic fluid has long been appreciated. The levels of several proteins, biomarkers for genetic malformations, have been investigated in detail. It is not known whether PACAP occurs in the amniotic fluid.

Cervico-vaginal fluid originates from the vaginal wall itself, and from cervical mucus and salpingo-uterine fluid.[21,22] Proteomic analysis has revealed dozens of proteins and peptides that play important roles in the defense mechanisms exerted by the cervico-vaginal fluid. Since PACAP has well-known anti-inflammatory effects, we aimed to study the possible occurrence of PACAP in the cervico-vaginal fluid.

In addition to fluids of the reproductive system, we analyzed other body fluids, like saliva, nasal fluid and aqueous humor. Human saliva is secreted from multiple salivary glands including parotid, submandibular, sublingual and other minor glands lying in the oral mucosa. The presence of PACAP has been shown in the main salivary glands of rodents.[23–25] It has been shown that PACAP enhances salivary flow and protein output and inhibits Ca^{2+} channels in the salivary glands.[24,26,27] Based on these observations, we aimed to examine whether PACAP is found also in the product of the salivary glands.

Human nasal fluid is produced by the epithelium of the nasal cavity and the underlying secretory glands. Proteomic analysis has identified 83 proteins in the nasal mucus of healthy adult volunteers using two-dimensional gel electrophoresis, MALDI-TOF and RPLC.[28] The presence of PACAP-containing nerve fibers has already been identified in the nasal mucosa, and PACAP has been shown to increase nasal airway resistance.[29] In addition, one study has suggested that PACAP could be a candidate biomarker of chronic sinusitis.[30] Therefore, we tested whether PACAP is present in the nasal mucosal fluid.

Human aqueous humor is a plasma filtrate from the blood vessels of the ciliary processes. It maintains intraocular pressure and plays an important role in the pathogenesis of ocular diseases. VIP, the peptide with closest structural similarity to PACAP, has been identified in human aqueous humor, showing significant differences between glaucoma and cataract patients.[30] However, it is not known whether PACAP occurs in this fluid. Therefore, the aim of our present study was to identify the presence of PACAP in human aqueous humor.

In summary, the aim of the present study was to determine the presence of PACAP in fluids of the female reproductive system, such as follicular and amniotic fluids and cervico-vaginal smear, and in further biological samples, such as saliva, nasal fluid and aqueous humor.

Materials and Methods

Biological samples

Human biological samples were collected according to a protocol approved by the institutional ethic committee (3117/2008, 3610/2009), during ophthalmological surgery or routine gynecological examinations, without extra intervention. Patients provided written approval of the sample collection in all cases. The samples were further processed for MS analysis based on modifications of earlier descriptions.[16] The peptidase inhibitor aprotinin was added to all samples (30 µl/ml), except for the cervico-vaginal fluid, nasal fluid and saliva on filter paper.

Follicular fluid was collected from female volunteers (aged between 20 and 35, $n = 40$) by follicular puncture after controlled ovarian hyperstimulation during the in vitro fertilization procedure.

Amniotic fluid specimens were collected at the 16th week of gestation from volunteering pregnant females undergoing amniocentesis as a prenatal diagnostic tool due to maternal age (age over 35 years, $n = 25$).

Cervico-vaginal fluid samples were collected from female volunteers in the progesterone phase of the cycle (aged between 25 and 35, $n = 10$) by the application of sterile filter paper strips (Schirmer paper) during colposcopic examination. Human nasal fluid (aged between 20 and 40, $n = 10$) and saliva (aged between 20 and 40, $n = 10$) were also collected by the application of sterile filter paper strips (Schirmer paper) from healthy volunteers.

Human aqueous humor was collected from volunteers (aged between 60 and 85, $n = 10$) during cataract surgery.

Sample preparation

A 100-µl of the follicular fluid sample was centrifuged at 10 000 rpm for 5 min, followed by the addition of 10 µl of 72% trichloroacetic acid and 100 µl of H_2O_2 to 90 µl of the supernatant. The samples were centrifuged at 13 000 rpm for 10 min after precipitation. The amniotic fluid (200 µl) was centrifuged at 10 000 rpm for 5 min. The supernatant (100 µl) was acidified by 100 µl 1% trifluoroacetic acid (TFA) and then centrifuged at 13 000 rpm for 10 min.

The solutions from the above-described samples were desalted and cleaned using 0.1% TFA solution with $ZipTip_{18}$ pipette tips (Millipore Kft., Hungary). The purified proteins and peptides were eluted directly onto the MALDI target plate (MTP 384 massive target T, Bruker Daltonics, Bremen, Germany) by 3 µl of acetonitrile/0.1% TFA (50 : 50, v/v) solution by mixing 1 µl of a saturated matrix solution, prepared freshly every day by dissolving a-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% TFA (1 : 2, v/v).

The cervico-vaginal fluid, human nasal fluid and saliva were dissolved by using 100 µl of acetonitrile–0.1% TFA (5 : 95, v/v) mixture in an ultrasonic bath at 5 min. The samples, including the human aqueous humor, were loaded onto the target plate (MTP 384 massive target T, Bruker Daltonics) directly by mixing 1–1 µl of each solution with the same volume of a saturated matrix solution, prepared freshly every day by dissolving a-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% TFA (1 : 2, v/v).

Mass spectrometry

Identification of PACAP38 was performed with MALDI TOF/TOF MS. Briefly, the mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics) operated in the linear detector for MALDI TOF or LIFT mode for high-energy collision-induced decay MALDI TOF/TOF with an automated mode using the FlexControl software. The ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with an acceleration voltage of 20.00 kV. The instrument uses a 337-nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH, Berlen, Germany). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206 195 Peptide Calibration Standard, Bruker Daltonics). Protein masses were acquired with a range of m/z 1000 to 10 000. Each spectrum was proceeded by accumulating data from 200 consecutive laser shots for standard PACAP38 solution and 1000 for amniotic, follicular, cervico-vaginal, nasal fluid, saliva and aqueous humor samples. The Bruker

FlexControl 2.4 software was used to operate the instrument and the Bruker Flexanalysis 2.4 software for spectrum evaluation.

Radioimmunoassay

Follicular fluid was collected as described above. The samples were weighed and centrifuged (12 000 rpm, 4 °C, 30 min), and the supernatant was further processed for RIA analysis of PACAP38-like immunoreactivity, as previously described.[16] Briefly, the conditions were as follows: antiserum: PACAP38: '88 111-3' (working dilution 1 : 10 000), tracer: mono-^{125}I-labeled ovine PACAP24-38 prepared in our laboratory (5000 cpm/tube), standard: ovine PACAP38 was used as a RIA standard ranging from 0 to 1000 fmol/ml, buffer: the assay was prepared in 1 ml of 0.05 mol/l (pH 7.4) phosphate buffer containing 0.1 mol/l sodium chloride, 0.25% (w/v) BSA and 0.05% (w/v) sodium azide. Incubation time: 48–72 h incubation at 4 °C. Separation solution: charcoal/dextran/milk powder (10 : 1 : 0.5 g in 100 ml distilled water).

Results

Mass spectrometry

The sample preparation procedure optimized for each biological fluid was suitable for measuring and identifying low molecular weight peptides by MS. Based on our previous and current results, sensitive and reproducible identification of PACAP38 can be carried out using linear MALDI TOF MS. The characteristic peak of PACAP38 is *m/z* 4536.0, as verified in the PACAP standard solutions (Fig. 1(a)). The collision-induced decay MALDI TOF/TOF fragmentation of PACAP38 standard yielded mainly *y* fragment ions of the PACAP38 parent ion (*m/z* 4536.0; Fig. 1(b)).[16] MS analysis revealed that PACAP38 was present in all of the stimulated ovarian follicular fluid samples. The mass spectrum of a representative positive follicular fluid sample with the characteristic PACAP38 peak is shown in Fig. 1(c). For further evidence of the presence of PACAP38 in the follicular fluid samples, the collision-induced decay MALDI TOF/TOF fragmentation was carried out in each case. This yielded similar fragments as found in PACAP38 standard (Fig. 1(d)). On the contrary, MS results could not prove the presence of the unmodified PACAP38 in any of the amniotic fluid, cervico-vaginal fluid, nasal fluid, human saliva or aqueous humor samples (from all these spectra with no identifiable PACAP peak, only one example is shown in Fig. 1(e)). The signal-to-noise ratios were also calculated in order to provide additional piece of evidence for the specificity of the peak representing PACAP38. These data are shown in Table 1.

Radioimmunoassay

PACAP38-like immunoreactivity was detected in the follicular fluid using a specific and sensitive RIA method. Average level of PACAP38 in follicular fluid samples ($n = 24$) was 161.1 ± 20.3 fmol/ml.

Discussion

The present study provided mass spectrometric evidence that PACAP occurs in the ovarian follicular fluid, while it is absent in the amniotic fluid, in the cervico-vaginal smear, nasal fluid, human saliva and in the aqueous humor.

In this study, we found the presence of PACAP38 in ovarian follicular fluid, obtained from 40 stimulated female patients. Here, all of the samples contained PACAP38. The follicular fluid serves as a culture medium for the developing oocyte, and it is important for the morphological and functional integrity for the germ cell development. PACAP has been shown to have several functions in follicular development. PACAP is expressed stage-specifically in granulosa cells of large mature follicles before ovulation, but weaker expression has also been shown in the wall of immature antral and pre-antral follicles.[19,32,33] PACAP receptors have also been demonstrated in developing follicles.[34–36] Both PACAP and PAC1 receptors have been demonstrated in the corpus luteum.[37] The PACAP found in the follicular fluid may derive from granulose and/or theca cells. The peptide is thought to play a role in primordial germ cell proliferation,[38] cyclic recruitment of immature follicles,[32] follicular apoptosis,[14,36] meiotic maturation of the oocytes[18] and ovarian hormone and enzyme production.[39–42] The finding that PACAP occurs in all of the ovarian follicular fluid samples indicates an important biological role for PACAP in this culture medium for the developing oocytes, the exact determination of which awaits further investigation. In addition, RIA measurements provided evidence for the presence of PACAP-like immunoreactivity in the follicular fluid samples. The variation in the immunoreactivity may be due to different physiological status or pathological conditions, the detailed analysis of which is beyond the scope of the present paper, but it could serve as a basis for further studies in the attempt to find correlation between pathological conditions, and/or the number of oocytes and PACAP levels.

In amniotic, cervico-vaginal, nasal fluid, saliva and in the aqueous humor we could not detect unmodified PACAP38 by MS. This could possibly be due either to the complete lack of PACAP in the samples, or levels below detection limit. The rapid digestion can be excluded as explanation, since we added peptidase inhibitor to the samples. It is also conceivable that in some samples PACAP, or its fragments, would occur in a modified form, the determination of which would require further experiments. Another possibility is that the different biological fluids exhibit different qualitative and quantitative composition. This could also be reflected in suppression effects, which is often observed in MALDI spectra of complex protein–peptide mixtures. The lack of PACAP found in the present study does not necessarily exclude the possibility that PACAP could be found under pathological circumstances. However, based on the present findings, we can conclude that under physiological circumstances PACAP, in its original form, is not present in the amniotic, cervico-vaginal, nasal fluid, saliva or in the aqueous humor.

In summary, our present data show, for the first time, the presence of PACAP38 in ovarian follicular fluid. The exact physiological role of PACAP in this biological fluid is not yet known, but based on the effects of PACAP in oogenesis, as well as in ovarian hormonal secretion, the peptide present in follicular fluid might play a role in oocyte function.

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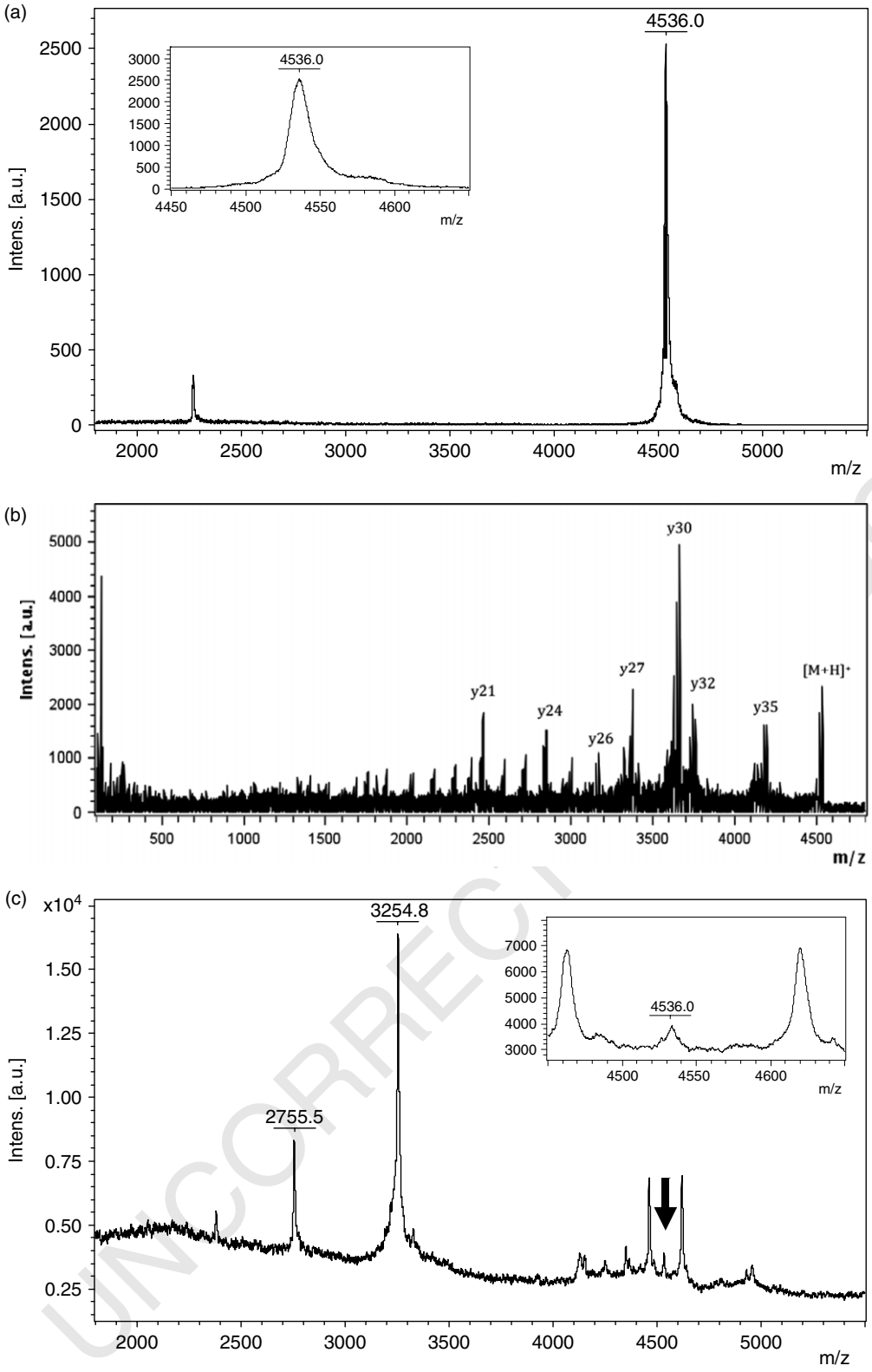

Figure 1. (a) MALDI TOF spectrum of PACAP38 in positive ion mode using linear detection indicating the protonated quasimolecular ion peak at *m/z* 4536.0 in PACAP38 standard. (b) Collision-induced decay fragmentation of the *m/z* 4536 peak as a parent ion with the main fragment *y* ions in PACAP38 standard. (c) MALDI TOF spectrum of follicular fluid samples. The peptide peak characteristic for PACAP38 (*m/z* 4536.0) could be identified in all of the samples. (d) Collision-induced decay fragmentation of the *m/z* 4536 peak as a parent ion with the main fragment *y* ions in follicular fluid samples. (e) Characteristic MALDI TOF spectrum of human saliva samples in positive ion mode using linear detection, where the characteristic peak of PACAP38 (*m/z* 4536.0) could not be found. Insets show the closer view of *m/z* range where the characteristic PACAP38 peak could be found.

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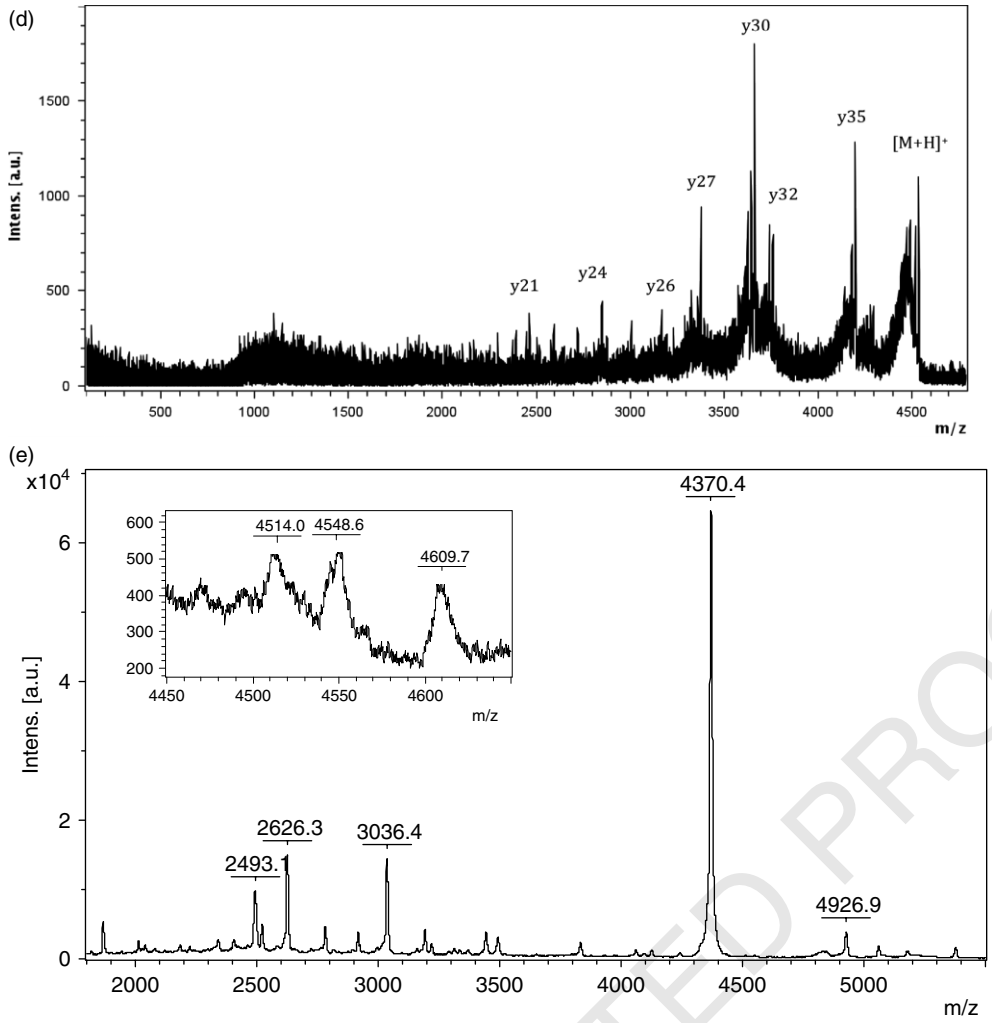

Figure 1. (*Continued*).

Table 1. The calculated signal-to-noise ratios of the samples

Sample	Peak intensity (*m/z* 4536)	Noise intensity	*S/N*
PACAP 38 standard	2130	35	61
Follicular fluid	920	45	20
Saliva	43	40	1

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AQ2

QUERIES TO BE ANSWERED BY AUTHOR

IMPORTANT NOTE: Please mark your corrections and answers to these queries directly onto the proof at the relevant place. Do NOT mark your corrections on this query sheet.

Queries from the Copyeditor:

AQ1 We have provided ''Investigation of pituitary adenylate cyclase activating polypeptide'' as the ''Running head'' for this article. It will appear on the top of the third page and all the recto pages of the article. Please confirm if this is appropriate.

AQ2 Refs [20] and [31] are not cited in the text, please check.

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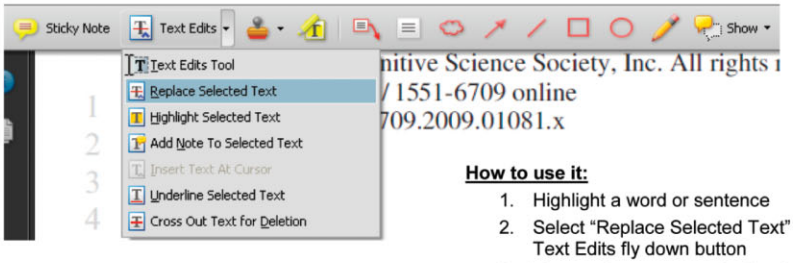
****(If the above toolbar does not appear automatically go to *Tools>Comment & Markup>Show Comment & Markup Toolbar*)****

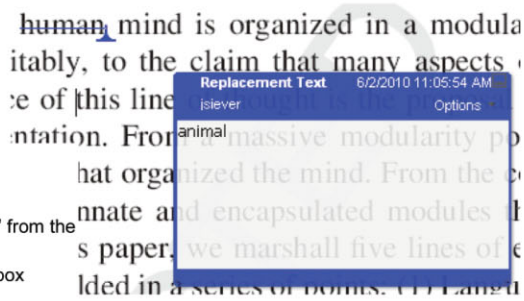
1. Replacement Text Tool — For replacing text.

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



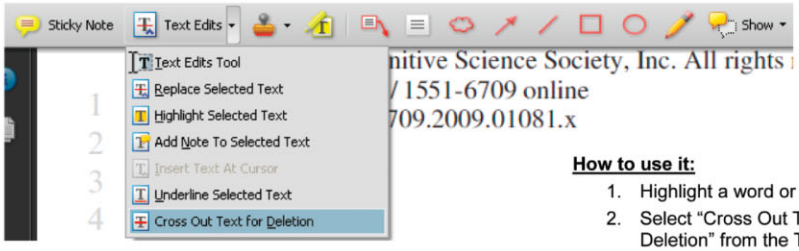
How to use it:

1. Highlight a word or sentence
2. Select "Replace Selected Text" from the Text Edits fly down button
3. Type replacement text in blue box



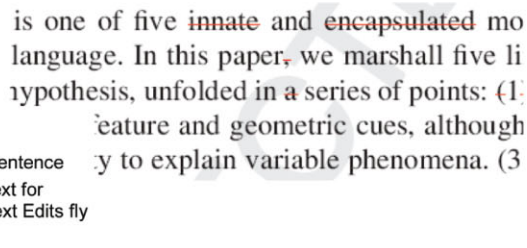
2. Cross-out Text Tool — For deleting text.

Strikes a red line through selected text.



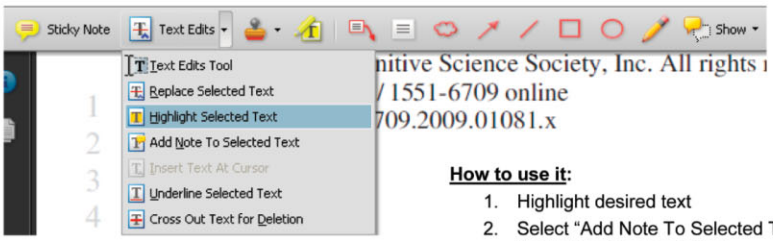
How to use it:

1. Highlight a word or sentence
2. Select "Cross Out Text for Deletion" from the Text Edits fly down button



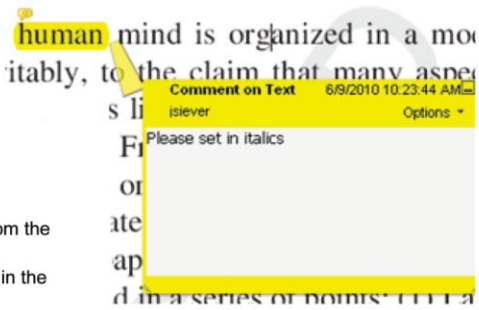
3. Highlight Tool — For highlighting a selection to be changed to bold or italic.

Highlights text in yellow and opens up a text box.



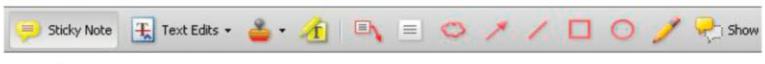
How to use it:

1. Highlight desired text
2. Select "Add Note To Selected Text" from the Text Edits fly down button
3. Type a note detailing required change in the yellow box



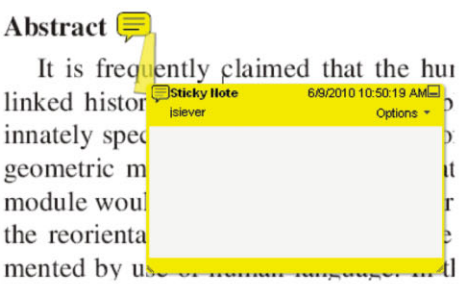
4. Note Tool — For making notes at specific points in the text

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



How to use it:

1. Select the Sticky Note icon from the commenting toolbar
2. Click where the yellow speech bubble symbol needs to appear and a yellow text box will appear
3. Type comment into the yellow text box

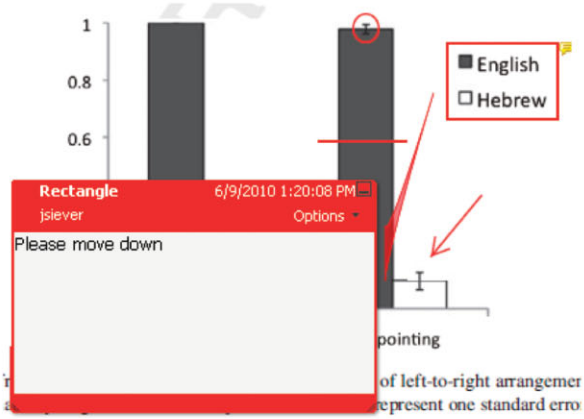


5. Drawing Markup Tools — For circling parts of figures or spaces that require changes

These tools allow you to draw circles, lines and comment on these marks.

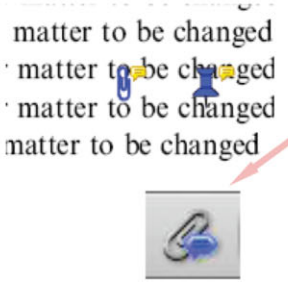
How to use it:

1. Click on one of shape icons in the Commenting Toolbar
2. Draw the selected shape with the cursor
3. Once finished, move the cursor over the shape until an arrowhead appears and double click
4. Type the details of the required change in the red box



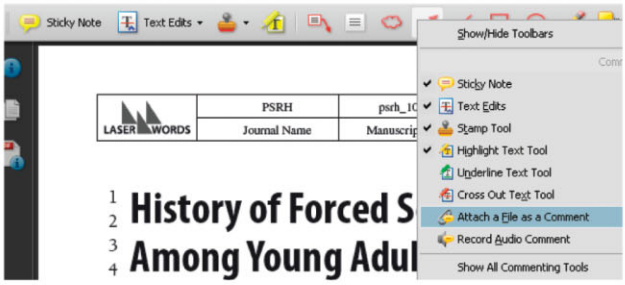
6. Attach File Tool — For inserting large amounts of text or replacement figures as a files.

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

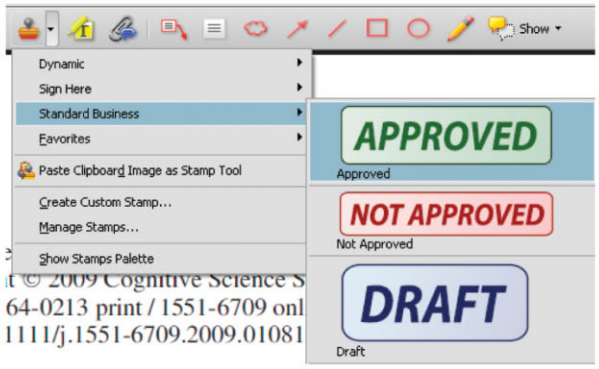


How to use it:

1. Right click on the Commenting Toolbar
2. Select "Attach a File as a Comment"
3. Click on paperclip icon that appears in the Commenting Toolbar
4. Click where you want to insert the attachment
5. Select the saved file from your PC or network
6. Select type of icon to appear (paperclip, graph, attachment or tag) and close

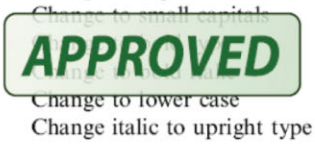


7. Approved Tool (Stamp) — For approving a proof if no corrections are required.



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)



Change to lower case
Change italic to upright type

Help

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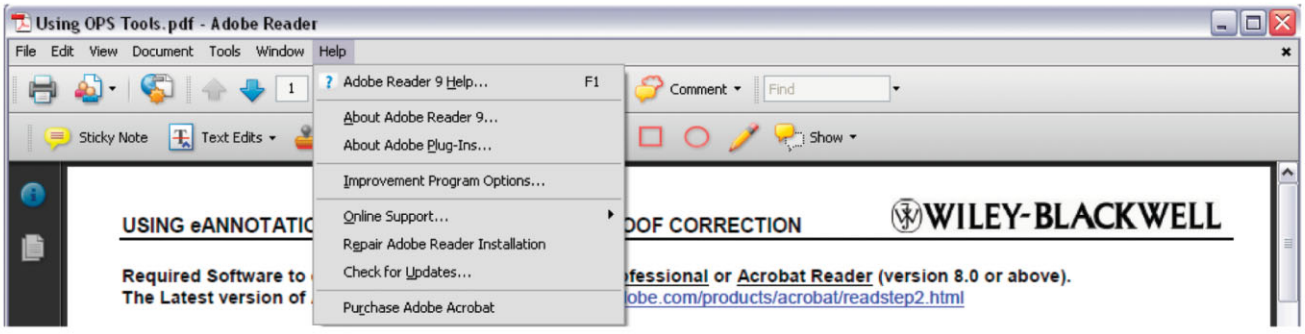

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