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SUMO1 in human sperm: new targets, role in motility and morphology and relationship with DNA damage

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Abstract

In studies carried out previously, we demonstrated that small ubiquitin-like modifier 1 (SUMO1) is associated with poor sperm motility when evaluated with a protocol that reveals mostly SUMO1-ylated live sperm. Recently, with another protocol, it has been demonstrated that SUMO is expressed in most sperm and is related to poor morphology and motility, suggesting that sumoylation may have multiple roles depending on its localisation and targets. We show herein, by confocal microscopy and co-immunoprecipitation, that dynamin-related protein 1 (DRP1), Ran GTPase-activating protein 1 (RanGAP1) and Topoisomerase IIα, SUMO1 targets in somatic and/or germ cells, are SUMO1-ylated in mature human spermatozoa. DRP1 co-localises with SUMO1 in the mid-piece, whereas RanGAP1 and Topoisomerase IIα in the post-acrosomal region of the head. Both SUMO1 expression and co-localisation with the three proteins were significantly higher in morphologically abnormal sperm, suggesting that sumoylation represents a marker of defective sperm. DRP1 sumoylation at the mid-piece level was higher in the sperm of asthenospermic men. As in somatic cells, DRP1 sumoylation is associated with mitochondrial alterations, this protein may represent the link between SUMO and poor motility. As SUMO pathways are involved in responses to DNA damage, another aim of our study was to investigate the relationship between sumoylation and sperm DNA fragmentation (SDF). By flow cytometry, we demonstrated that SUMO1-ylation and SDF are correlated ($r=0.4$, $P<0.02$, $n=37$) and most sumoylated sperm shows DNA damage in co-localisation analysis. When SDF was induced by stressful conditions (freezing and thawing and oxidative stress), SUMO1-ylation increased. Following freezing and thawing, SUMO1–Topoisomerase IIα co-localisation and co-immunoprecipitation increased, suggesting an involvement in the formation/repair of DNA breakage.

Reproduction (2014) **148** 453–467

Introduction

Following release from the testis, sperm own a highly condensed DNA, and transcription and translation processes are silent. Therefore, post-translational modifications represent the main way for sperm to acquire their functionality (Blaquier *et al*. 1988*a*,*b*, Ross *et al*. 1990). Among post-translational modifications occurring in mature sperm (Muratori *et al*. 2011), protein sumoylation has been recently described (Marchiani *et al*. 2011, Vigodner *et al*. 2013). Sumoylation consists in the attachment of small ubiquitin-like modifier (SUMO) peptides via its C-terminal glycine residue to the lysine residues of the protein targets, mediated by E1, E2 and E3 enzymes (Geiss-Friedlander & Melchior 2007). Sumoylation plays a regulatory role in many cellular processes, including DNA damage control and regulation of mitochondrial dynamics (Geiss-Friedlander & Melchior 2007). Although extensively studied in somatic cells, little is known about protein sumoylation in testicular functions and mature sperm (Vigodner 2011). SUMO1 has been found in mouse and rat testis (Vigodner & Morris 2005, La Salle *et al*. 2008) in all stages of the process of spermatogenesis, and in specific chromatin and other cellular domains both in germ and somatic cells of human testis (Vigodner *et al*. 2006), but its role during spermatogenesis remains elusive. Recently, modifications in the levels of SUMO1 and SUMO2/3 have been detected in mouse germ cells following exposure to oxidative and heat stress (Shrivastava *et al*. 2010), suggesting a role in responses to stress, as described for somatic cells (Comerford *et al*. 2003, Manza *et al*. 2004). Interestingly, following stress stimuli, SUMO localised to the sites of DNA double-strand breaks (DSBs; Shrivastava *et al*. 2010). We have recently

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-14-0173
Online version via www.reproduction-online.org

demonstrated that, in ejaculated human sperm, SUMO1 is localised in the nucleus and, to a lesser extent, in the mid-piece (Marchiani *et al*. 2011). In addition, we found that when sumoylation is analysed using a short-time (4 min) permeabilisation protocol by an immunofluorescence/flow cytometric method, it is mostly detected in live sperm and the percentage of SUMO1-positive live sperm negatively correlates with progressive and total motility in asthenospermic men (Marchiani *et al*. 2011), indicating that sumoylation, when evaluated with such a protocol, may be a marker of immotile live sperm. However, when a long-time permeabilisation protocol was employed, a high percentage of sperm was found to express SUMO1 (Marchiani *et al*. 2011), in line with recent results (Vigodner *et al*. 2013) obtained with a different permeabilisation protocol where a relation between excessive sumoylation and abnormal sperm morphology was unmasked. Overall, these results indicate that sumoylation could be necessary (being present in most sperm) and, at the same time, deleterious (marking immotile and morphologically abnormal sperm) for human sperm functions depending on its extension, its localisation within the cell and, likely, on the specific proteins that are being SUMO-modified. In such a scenario, identification of targets will give insights in understanding the functions of the SUMO pathway in sperm. Although several SUMO2/3-ylated proteins were identified by mass spectrometry (Vigodner *et al*. 2013), the role of sumoylation of these proteins in sperm functions remains undefined.

One aim of this study was to identify SUMO1 target proteins and their possible involvement in sperm functions. In particular, in view of the prominent role of sumoylation in regulation of mitochondrial dynamics (Livnat-Levanon & Glickman 2011) and the role of this organelle in sperm motility, we focused our attention on dynamin-related protein 1 (DRP1), the main substrate of SUMO in somatic cell mitochondria (Harder *et al*. 2004, Zunino *et al*. 2007). Another possible SUMO sperm target is Ran GTPase-activating protein 1 (RanGAP1), the first documented substrate for SUMO in somatic cells, implicated in nuclear–cytoplasmic transport and in the organisation of microtubules in spermatids (Dasso 2001, Kierszenbaum *et al*. 2002), and hypothesised to be a SUMO target in germ cells (Vigodner 2011). Moreover, in view of its involvement in sperm chromatin remodelling during spermiogenesis (McPherson & Longo 1993), we investigated sumoylation of Topoisomerase IIα, identified as a SUMO target in mice germ cells (Shrivastava *et al*. 2010).

Finally, in view of emerging data documenting implication of SUMO in responses to generation of DNA DSBs (Bekker-Jensen & Mailand 2011) and that SUMO1, SUMO2/3, and sumoylating enzymes UBC9, PIAS and E3s accumulate at the sites of DSBs (Galanty *et al*. 2009, Morris 2010, Shrivastava *et al*. 2010), another aim of the study was to investigate the relationship between sperm sumoylation and sperm DNA fragmentation (SDF), whose role in male infertility is increasingly recognised (Tamburrino *et al*. 2012).

Materials and methods

Chemicals

Human tubal fluid (HTF) medium and human serum albumin (HSA) were purchased from Technogenetics (Milan, Italy). Diff-Quick Kit was purchased from CGA, Diasint (Florence, Italy). BSA was purchased from ICN Biomedicals (Irvine, CA, USA). Monoclonal mouse antibody anti-SUMO1 (21C7), monoclonal mouse antibody anti-RanGAP1 and Alexa Fluor 546 Goat Anti-Mouse IgG (H+L) were purchased from Invitrogen. Rabbit polyclonal antibody anti-DRP1 (H-300) was purchased from Santa Cruz Biotechnology, mouse monoclonal anti-DRP1 was from Becton Dickinson (Mountain View, CA, USA), and rabbit anti-Topoisomerase IIα and rabbit anti-SUMO2/3 were from Cell Signaling Technologies (Boston, MA, USA). Mouse IgG1a isotype control was purchased from Exbio (Praha, Czech Republic) and negative control rabbit serum was purchased from Signet Laboratories (Dedham, MA, USA). Goat anti-rabbit IgG (H+L) FITC was from Southern Biotech (Birmingham, AL, USA). Paraformaldehyde (PFA) was obtained from Merck Chemicals. The other chemicals were from Sigma Chemical.

Semen samples collection, preparation and treatment

Semen samples were collected, according to WHO criteria (World Health Organization 2010), from men undergoing routine semen analysis in the Andrology Laboratory of the University of Florence, after endorsement of Local Ethics Committee and patients' informed consent. Semen analysis was carried out according to WHO criteria (World Health Organization 2010). Sperm morphology was evaluated by determining the percentage of normal and abnormal forms after Diff-Quik staining, by scoring 200 sperms/slide. Sperm motility was scored by determining the percentage of progressively motile, non-progressively motile and immotile sperm from at least 100 sperms/slide. Immunofluorescence, confocal microscopy and flow cytometry experiments were performed in rough semen samples after washing twice with HTF medium and fixation with PFA (500 µl, 4% in PBS, pH 7.4) for 30 min at room temperature (RT). For immunoprecipitation and western blot analysis, sperm samples were selected by gradient separation. The semen samples were layered on a 40% fraction of PureSperm (Nidacon, Mölndal, Sweden), prepared in HTF/HSA medium and centrifuged at 500 ***g*** for 30 min at 26 °C. The resulting 40% pellet (representing the unselected fraction of sperm devoid of germ and other cells present in semen) was collected and washed in HTF/HSA medium. Each sample was carefully checked for effective elimination of germ cells and leucocytes and for maintenance of the initial motility conditions by microscope observation. Finally, the samples were washed twice in PBS and stored at −80 °C after addition of sodium orthovanadate.

Protein extraction and western blot analysis

Protein extraction and western blot analysis were carried out as described previously (Muratori *et al*. 2004, Luconi *et al*. 2005, Marchiani *et al*. 2011). Briefly, after selection by gradient separation (aforementioned), sperm samples were washed in PBS supplemented with 1 mM Na_3VO_4, centrifuged and resuspended in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.25% NP-40, 1 mM Na_3VO_4, 1 mM phenylmethyl-sulfonyl fluoride and 10 μg/ml leupeptin) with 20 mM *N*-ethylmaleimide (Sigma Chemical Co.), an inhibitor of all cysteine peptidases. After measurement of protein concentration (determined by Coomassie brilliant dye; Bio-Rad Laboratories), aliquots of cell lysates containing equal amount of proteins were resuspended in 20 μl of 2× Laemmli's reducing sample buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 2.5% pyronin and 200 mM dithiothreitol), boiled at 95 °C for 5 min and loaded onto 8% polyacrylamide–bisacrylamide gels. In some experiments, pre-casted gels were used (Bio-Rad Laboratories).

After SDS–PAGE, proteins were transferred to nitrocellulose membrane and incubated with the specific primary antibodies (anti-SUMO1, anti-SUMO2/3, anti-DRP1, anti-RanGAP1 and anti-Topoisomerase IIα at the indicated concentrations) overnight in 1% BSA blocking buffer in TTBS solution (Tris-buffered saline (TBS) containing 0.1% Tween 20, pH 7.4), washed and incubated with peroxidase-conjugated relative secondary antibodies (1:2000) for 2 h. As control for lane loading, membranes were re-probed with anti-β-actin primary antibody. Expression of proteins was revealed by Immobilon Western, chemiluminescent HRP substrate (Millipore, Bedford, MA, USA). Image acquisition was performed using Quantity One Software on a ChemiDoc XRS instrument (Bio-Rad Laboratories).

Magnetic immunoprecipitation

For immunoprecipitation experiments, Protein A Microbeads (MACS MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) were used, and magnetic immunoprecipitation has been carried out following the protocol suggested by the manufacturer. Briefly, primary antibodies (anti-SUMO1 (2 μg), anti-DRP1 (4 μg), anti-RanGAP1 (2 μg) or anti-Topoisomerase IIα (2.4 μg/μl)) were added to Protein A Microbeads and sperm lysates (SL; 1 mg of proteins) and incubated for 30 min on ice. The magnetically labelled immune complex was passed over a micro column placed in the magnetic field. The columns were washed to eliminate excessive proteins, whereas the magnetically labelled immune complex remains bound to the micro column and were eluted with Laemmli's reducing sample buffer for SDS–PAGE and western blotting analysis for DRP1, RanGAP1 and Topoisomerase IIα. For negative controls, sperm protein lysates were immunoprecipitated both with only beads and with mouse IgG1 (for SUMO1 and RanGAP1) or rabbit serum (for DRP1 and Topoisomerase IIα) at the same concentrations of the corresponding primary antibody.

Detection of SUMO1 by flow cytometry

For detection of SUMO1, samples were processed as previously described (Marchiani *et al*. 2011). Briefly, after fixation and washing twice with 1% normal goat serum (NGS)–PBS, sperm were permeabilised with 0.1% Triton X-100 in 100 μl of 0.1% sodium citrate for 4 min in ice. Then, samples were split into two aliquots subsequently incubated (1 h at room temperature (RT)) either with anti-SUMO1 antibody (10 μg/ml, in 1% NGS–PBS, test sample) or with anti-mouse IgG1 (10 μg/ml, in 1% NGS–PBS, isotype control). After washing twice with 1% NGS–PBS, sperm were incubated in the dark for 1 h with FITC-conjugated goat anti-mouse IgG (dilution 1:100 in 1% NGS–PBS). The samples were washed twice, resuspended in 500 μl of PBS, stained with 10 μl of Propidium iodide (PI, 30 μg/ml in PBS) and incubated in the dark for 15 min at RT. An additional sperm suspension was prepared with the same procedure for test sample, but omitting the PI staining, and used for instrumental compensation. The samples were acquired using FACScan flow cytometer (Becton Dickinson) equipped with a 15-mW argon ion laser for excitation. Green fluorescence of FITC-conjugated goat antimouse IgG was revealed by an FL-1 (515–555 nm wavelength band) detector; red fluorescence of PI was detected by an FL-2 (563–607 nm wavelength band) detector. We determined sperm SUMO1 positivity within the nucleated events (i.e. the events labelled with PI) of the characteristic forward scatter/side scatter (FSC/SSC) region of sperm (Muratori *et al*. 2008). The amount of sperm with SUMO1 positivity was scored as the percentage of sperm having fluorescence intensities above a threshold excluding ≤1% of the events in the isotype control. CellQuest-Pro Software program (Becton Dickinson) was used for acquisition and analysis.

Immunofluorescence and confocal microscopy

The protocol was similar to that described for the detection of SUMO1 by flow cytometry (aforementioned) with the difference that incubation with primary antibodies (anti-SUMO1, anti-SUMO2/3, anti-DRP1, anti-RanGAP1 and anti-Topoisomerase IIα) was performed in 0.1% Triton X-100 in 100 μl of 0.1% sodium citrate, for 1 h at 37 °C. Moreover, secondary antibodies were different with respect to immunofluorescence protocol: FITC-labelled anti-rabbit IgG (1:100) was used to detect SUMO2/3, DRP1 and Topoisomerase IIα expression, FITC-labelled anti-mouse IgG (1:100) for RanGAP1 expression and AlexaFluor 546-conjugated anti-mouse IgG (1:200) for SUMO1 expression.

After labelling, the sperm were smeared on slides and mounted using an antifade mounting medium (ProLong Gold Antifade Reagent, Invitrogen). The samples were observed under a Leica TCS SP5 laser scanning microscope (Leica Microsystems, Mannheim, Germany), equipped with a HeNe/Ar laser source, using a Leica Plan Apo ·63/1.40 NA oil immersion objective. The series of optical sections (1024×1024 pixels each; pixel size 200×200 nm) were taken through the depth of the cells at intervals of 0.4 lm. The 3D confocal stacks were then projected as a single composite image by the maximum intensity method. To reduce bleed-through effects, dual channel scanning of red and green signals were recorded separately and saved in two different files. For examination of samples stained with SUMO2/3 a fluorescence microscope (Carl Zeiss, Axiolab A1 FL, Milan, Italy), equipped with Filter 15 and oil immersion 100× magnification objective was used.

Immunoelectron microscopy

The semen samples were fixed for 2 h at 4 °C in 4% PFA/0.5% glutaraldehyde in PBS. Then, the samples were dehydrated in a graded series (50, 75, 95 and 100%) of EtOH and infiltrated first with 1:1 EtOH/100% London White Resin (Hard Grade Catalysed; TAAB Laboratories, England, UK) overnight at 4 °C and next with pure resin, at 4 °C overnight. Finally, the samples were embedded in pure resin and polymerised at 50 °C for 24 h. Ultrathin sections were cut by a Supernova Reichert-Jung ultramicrotome (Wien, Austria), collected on gold grids and incubated at RT for 20 min with glycine (0.02 m) in PBS. After washing in PBS, the sections were saturated for 20 min with TBS–1% BSA containing 5% NGS and treated overnight at 4 °C with mouse anti-SUMO1 antibody and with anti-DRP1 antibody diluted 1:20 in TBS–2% NGS. After incubation, the sections were washed six times for 5 min in TBS and then incubated overnight at 4 °C with the goat anti-mouse IgG 20 nm gold conjugated and goat anti-rabbit IgG 10 nm gold-conjugated secondary antibodies, both diluted 1:50 in TBS–2% NGS. After extensively washing in TBS, the sections were post-fixed in 3% glutaraldehyde in 0.06 m cacodylate buffer. After deep rinsing in TBS (6×2 min) followed by distilled water, the grids were stained with uranyl acetate. Observations and micrographs were made using a FEI Tecnai (Hillsboro, OR, USA) transmission electron microscope.

Evaluation of SDF

We evaluated SDF by using TUNEL/PI assay recently set up in our laboratory (Muratori *et al.* 2010). Briefly, 10×10^6 sperm were fixed in 4% PFA and immediately processed for TUNEL labelling. The sperm were centrifuged at 500 ***g*** for 10 min and washed twice with 200 µl of PBS with 1% BSA. Then the sperm were permeabilised with 0.1% Triton X-100 in 100 µl of 0.1% sodium citrate for 4 min in ice. After washing two times, the labelling reaction was carried out by incubating sperm in 50 µl of labelling solution (supplied with the *In Situ* Cell Death Detection Kit, fluorescein, Roche Diagnostics) containing the TdT enzyme, for 1 h at 37 °C in the dark. Finally, the samples were washed twice, resuspended in 500 µl of PBS, stained with 10 µl of PI (30 µg/ml in PBS) and incubated in the dark for 15 min at RT. For each test sample, a negative control (omitting TdT) and a sample for fluorescence compensation (labelled only with TUNEL) were prepared. We determined SDF within the nucleated events (i.e. the events labelled with PI) of the characteristic FSC/SSC region of sperm (Muratori *et al.* 2008). Green fluorescence (of nucleotide conjugated with fluorescein) and red fluorescence (of PI) were revealed, respectively, by the FL-1 (515–555 nm wavelength band) and the FL-2 (563–607 nm wavelength band) detectors of a FACScan flow cytometer (Becton Dickinson). We calculated the percentage of DNA fragmented sperm within the PI-positive events of the R1 region (Muratori *et al.* 2008).

Evaluation of SDF and SUMO1 after freezing and thawing and H_2O_2 treatment

Freezing and thawing of sperm was performed as described previously (Meamar *et al.* 2012). Briefly, each semen sample ($n=6$) was divided into four aliquots. Two aliquots (each contained 10×10^6 sperm) were used to determine pre-freezing SDF and SUMO1-ylation in fixed samples. The remaining two aliquots were used for cryopreservation in Test Yolk Buffer (TYB). The sperm were frozen in liquid nitrogen in a Taylor-Wharton 34HC tank (Taylor-Wharton, Theodore, AL, USA) by manually controlled freezing procedure. Briefly, samples were diluted 1:1 (vol:vol) by adding TYB dropwise. After equilibration at RT for 10 min, the sperm were loaded in 500 µl straws. The straws were frozen by exposure to liquid nitrogen vapours for 8 min and finally plunge into liquid nitrogen for 1 h, according to Taylor-Wharton procedure. Thawing was carried out by transferring the straw at RT for 10 min and then at 37 °C for 10 min. The same treatment was used for IP experiments evaluating SUMO1-ylation of Topoisomerase IIα and for SUMO1 being evaluated by western blotting analysis of total SL. The washed semen samples were treated with H_2O_2 (5 mM) by incubating the sperm for 2 h at 37 °C according to Smith *et al.* (2013).

After washing, treated (by freezing and thawing and by H_2O_2) and untreated sperm samples were fixed in 4% PFA and immediately processed for the detection of TUNEL/PI and SUMO1 (as described earlier). Sperm viability (as percentage of live sperm) was calculated before and after freezing and thawing and H_2O_2 treatment by eosin–nigrosin staining (World Health Organization 2010).

Simultaneous detection of SDF and SUMO1 in sperm

To study the simultaneous detection of SDF and SUMO1 in sperm, we carried out a triple labelling reaction: i) with TUNEL–Fluorescein to reveal SDF; ii) with immunofluorescence technique for SUMO1 and iii) with DAPI. After fixation in 4% PFA, the samples were processed for the detection of SUMO1, using the same procedure described earlier, except for the secondary antibody that was conjugated with AlexaFluor 546 instead of FITC, and then for TUNEL, with the procedure described earlier, except that samples were incubated with DAPI (1 µg/ml) at 37 °C for 15 min instead of PI. The samples were acquired using FACSAria flow cytometer (Becton Dickinson) equipped with blue, red and violet lasers for excitation at 488 nm (for FITC and AlexaFluor 546) and 405 nm (for DAPI) lasers. Blue (DAPI), green (FITC) and red (AlexaFluor 546) fluorescence were revealed by PTMs photomultipliers equipped with 450/40, 530/30 and 585/42 BP filters respectively. Acquisition and analysis were performed using FACSDiva Software (Becton Dickinson). Double-stained samples for SUMO1 and TUNEL were smeared on slides for examination under fluorescence microscope.

Statistical analyses

All statistical analyses were carried out using the SPSS version 20 Software for Windows (SPSS, Inc.). The distribution of each parameter was tested for normality by Kolmogorov–Smirnov test. Data are expressed as mean±S.D. and statistical comparisons were made using Student's *t*-test. Bivariate correlation between SDF and sumoylation was evaluated by calculating the Pearson's correlation coefficient.

Results

As mentioned earlier, identification of SUMO protein targets represents an essential step in understanding the possible functions of sumoylation in sperm. Herein, we investigated whether DRP1, Topoisomerase IIα and RanGAP1, known to be SUMO substrates in somatic or germ cells, are also target for SUMO in human sperm.

Dynamin-related protein 1

DRP1 is a mitochondrial protein involved in fission and fusion processes of this organelle (Otera *et al*. 2013). Studies carried out in somatic cells showed that persistent SUMO1 modification of DRP1 in mitochondria leads to morphological alterations and fragmentation of the organelle (Harder *et al*. 2004, Zunino *et al*. 2007). Mitochondria integrity and functionality are of fundamental importance for sperm motility (Paoli *et al*. 2011, Amaral *et al*. 2013).

To investigate whether DRP1 is SUMO1-ylated, sperm were labelled both for SUMO1 and DRP1 and observed by confocal laser microscopy. As shown in Fig. 1A, a green signal, corresponding to unSUMO1-ylated DRP1 was observed in the principal or the end piece of the tail (white arrows) and, in few sperm, in the head. DRP1 positivity was observed in about 60% spermatozoa. SUMO1 (red signal) was mostly localised in the head, neck and mid-piece (Fig. 1A). A yellow signal indicating the co-localisation of the two proteins was frequently present at the mid-piece level (yellow arrows), where the mitochondria are located. Occasionally, co-localisation of the two proteins was observed also in the tail, and, more sporadically, in the head (Fig. 1A). These data suggest that DRP1 may be SUMO1-ylated in human sperm. To confirm DRP1 SUMO1-ylation, we performed immunoprecipitation experiment with anti-SUMO1 or anti-DRP1 followed by western blotting analysis respectively with anti-DRP1 and anti-SUMO1 antibodies. In 40% PureSperm selected SL blotted with anti-DRP1 antibody, a band of ∼80 kDa, corresponding to the molecular weight of DRP1, and other bands at a higher molecular weight (∼90 and ∼150 kDa) were present (Fig. 1B, SL). Both bands were present in the lysates of pooled sperm from asthenospermic (SL:A) and normospermic patients (SL:N). The two bands (∼90 and

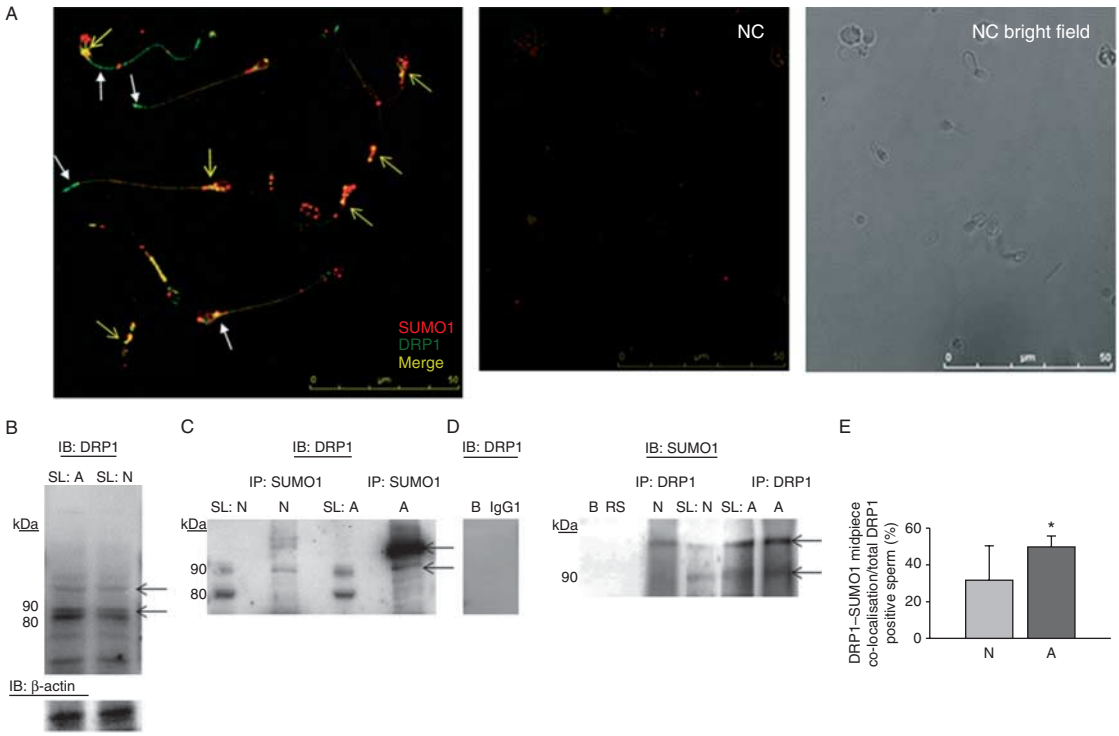

Figure 1 Identification of SUMO1-ylated DRP1 in human sperm. (A) Images obtained by immunofluorescence confocal laser microscopy of DRP1 (green signal, white arrows) and SUMO1 (red signal). DRP1-positive spermatozoa were selected to compose the image. Yellow signal indicates co-localisation of the two proteins. Yellow arrows indicate co-localisation of DRP1 and SUMO1 in the mid-piece. Negative control (NC) and the relative bright field (NC bright field) are shown in middle and right panels. (B, C and D) Western blotting analysis of 40% PureSperm-selected sperm lysate (SL, B) and immunoprecipitates with anti-SUMO1 antibody (IP:SUMO1, C) or anti-DRP1 (IP:DRP1, D) of pooled SL from asthenospermic (A) and normospermic (N) subjects, blotted with anti-DRP1 and anti-SUMO1 antibodies respectively. Negative controls have been performed using only beads (B) and mouse IgG1 (IgG1 for SUMO1, C) or rabbit serum (RS for DRP1, D). Black arrows indicate the bands corresponding to SUMO1-ylated DRP1 and images are representative of three similar experiments for SL and two for IP. The membrane of SL shown on the left was also blotted with anti-β-actin as loading control. (E) Histograms reporting mean (±S.D.) percentage of sperm with DRP1–SUMO1 mid-piece co-localisation on the total of DRP1 positive in six normo- and seven asthenospermic subjects. *P=0.04 vs normospermic subjects.

~150 kDa) were also present following immunoprecipitation with anti-SUMO1 antibody and blotting with anti-DRP1, both in IP from normo- (N) and asthenospermic (A) men (Fig. 1C, IP:SUMO1), suggesting that they correspond to SUMO1-conjugated DRP1. Both bands were also present after immunoprecipitation with anti-DRP1 antibody and blotting with anti-SUMO1 in pooled sperm of asthenozoospermic subjects (Fig. 1D, IP:DRP1). In the anti-DRP1 IP of pooled samples from normospermic men, despite the presence of both bands in total lysates, the ~90 kDa band was not evident. Such experiment has been repeated several times with two different anti-DRP1 antibodies with similar results. It is possible that the anti-DRP1 antibodies, although indicated also for IP by manufacturers, do not have high efficiency for this type of experiments.

To the best of our knowledge, this is the first study investigating the presence of DRP1 and its possible sumoylation in sperm, to gain further insights about the localisation of DRP1 and interaction with SUMO1, we have carried out electron microscopy experiments using gold granules of different size to reveal two proteins. Such experiments confirmed the localisation of DRP1 at tail level and reveal the co-presence of DRP1 and SUMO in tails and in mitochondria (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

As mentioned earlier, in previous studies (Marchiani *et al*. 2011, Vigodner *et al*. 2013) a relationship between sumoylation and sperm motility has been observed. To investigate whether DRP1/SUMO1 co-localisation at the mid-piece level is associated with decreased sperm motility, DRP1 and SUMO1 immunofluorescence in seven normospermic and six asthenospermic subjects was detected by confocal microscopy experiments, and the percentage of sperm showing co-localisation between SUMO1 and DRP1 at the mid-piece on the total DRP1 positive cells was calculated. A higher percentage of sperm showed co-localisation between the two proteins in the mid-piece in asthenospermic subjects compared with normospermic (mean$\pm$S.D.: 49.9$\pm$5.7% in asthenospermic and 32.4$\pm$18.3% in normospermic subjects, $P=0.04$; Fig. 1C). In addition, a significant negative correlation was found between total sperm motility and the percentage of sperm showing co-localisation between DRP1 and SUMO1 in the mid-piece ($r=-0.64$, $P=0.016$, $n=13$).

Topoisomerase IIα

Another possible nuclear target of SUMO1 in sperm is Topoisomerase IIα. Indeed, such enzyme, which is implicated in the remodelling of sperm chromatin during spermiogenesis (McPherson & Longo 1993, Laberge & Boissonneault 2005), has recently been shown to be a SUMO target in mouse spermatocytes (Shrivastava *et al*. 2010).

We found that Topoisomerase IIα is located (Fig. 2A, green signal, white arrows) and co-localised with SUMO1 (yellow signal, yellow arrows) in a discrete area within the post-acrosomal region of the sperm head. Interestingly, Topoisomerase IIα was present in most sperm (about 80% showed a positive intense green signal). Western blot experiments (Fig. 2B) showed the presence of two bands in total SL, including one of molecular weight of ~100 kDa, corresponding to that of Topoisomerase IIα in sperm (Har-Vardi *et al*. 2007) and one at ~110 kDa. Lack of a band at 170 kDa, corresponding to the somatic (Har-Vardi *et al*. 2007) and testicular (Shrivastava *et al*. 2010) form of Topoisomerase IIα indicates absence or little contamination of germ and somatic cells in the SL prepared by centrifugation in 40% PureSperm. After immunoprecipitation with anti-SUMO1 and anti-Topoisomerase IIα antibodies (Fig. 2B, IP:SUMO1 and IP:Topoisomerase IIα) and reciprocal blotting, a band of width of ~110 kDa, likely corresponding to the sumoylated form of the protein, was present.

To evaluate whether SUMO1-ylation of Topoisomerase IIα was involved in sperm motility as it occurs for DRP1, we correlated the percentage of spermatozoa with co-localisation between SUMO1 and Topoisomerase IIα with sperm motility in seven samples. No correlation or a trend to it was found ($r=-0.05$, $P=0.96$).

Ran GTPase-activating protein 1

Considering the massive localisation of SUMO1 in the sperm nucleus (Marchiani *et al*. 2011), we focused our attention on RanGAP1, a substrate of SUMO1 reported to be found in somatic cells, implicated in cytosol–nucleus transport. As for DRP1 and Topoisomerase IIα, sumoylation of RanGAP1 was investigated by confocal microscopy, immunoprecipitation and western blotting analysis (Fig. 3). As can be observed, RanGAP1 is localised in the head (Fig. 3A, green signal, white arrow) and was present in about 70% of sperm. Co-localisation between RanGAP1 and SUMO1 was frequently found in the post-acrosomal region of the sperm head (Fig. 3A, yellow arrows). Figure 3B shows the results of immunoprecipitation and western blotting analysis. As can be observed, two bands are present in SL blotted with anti-RanGAP1 antibody (Fig. 3B, left panel, SL), one at the molecular weight of ~70 kDa, corresponding to the molecular weight of RanGAP1, and an additional band at ~80 kDa. Following immunoprecipitation of pooled sperm samples, both with anti-SUMO1 (Fig. 3B, left panel, IP:SUMO1) and anti-RanGAP1 (Fig. 3B, right panel, IP:RanGAP1) antibodies and reciprocal blotting, one band appeared at ~80 kDa, likely corresponding to SUMO1-conjugated RanGAP1.

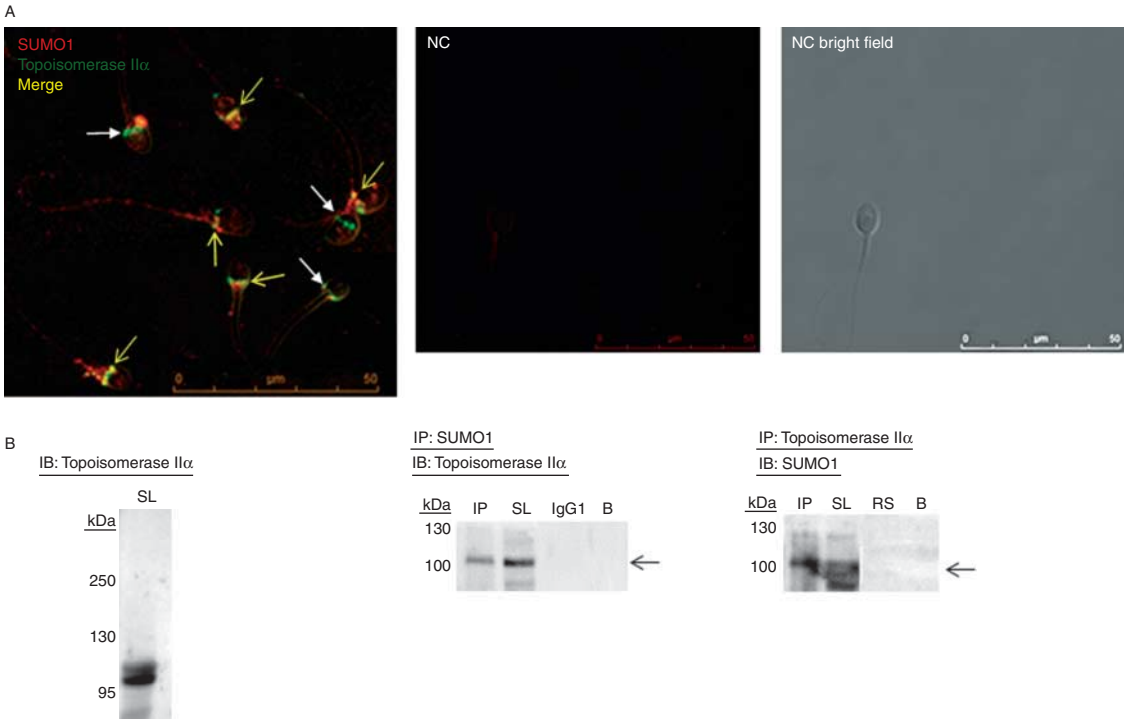

Figure 2 Identification of SUMO1-ylated Topoisomerase IIα in human sperm. (A) Images obtained by immunofluorescence confocal laser microscopy of Topoisomerase IIα (green signal, white arrows) and SUMO1 (red signal). Topoisomerase IIα-positive spermatozoa were selected to compose the image. Yellow signal indicates co-localisation of the two proteins (yellow arrows) in the post-acrosomal region of the head. Negative control (NC) and the relative bright field (NC bright field) are shown in the middle and right panels. (B) Western blotting analysis of 40% PureSperm-selected sperm lysates (SL) and after immunoprecipitation with anti-SUMO1 (IP:SUMO1) and anti-Topoisomerase IIα (IP:Topoisomerase IIα) antibodies, blotted respectively with anti-Topoisomerase IIα and anti-SUMO1 antibodies. The latter experiments have been carried out using pre-casted gels from Bio-Rad. Negative controls have been performed using only beads (B) and mouse IgG1 (IgG1 for SUMO1) or rabbit serum (RS for Topoisomerase IIα). Black arrows indicate the band corresponding to SUMO1-ylated Topoisomerase IIα. Results are representative of two similar experiments.

SUMO1-ylation in morphologically abnormal sperm

As mentioned in the introduction, Vigodner *et al.* (2013) reported an association between sumoylation and poor morphology, unrecognised in our previous study (Marchiani *et al.* 2011). Such discrepancy could be due to the different permeabilisation protocols used in the two studies. In this study, to better understand the relationship between sumoylation and sperm morphology, confocal microscopy experiments were performed using a long-time permeabilisation protocol. With such protocol, followed by flow cytometric analysis, we have previously shown that the great majority of sperm express SUMO1 (Marchiani *et al.* 2011). Next, we evaluated the percentage of SUMO1-positive sperm, showing apparently normal or abnormal morphology (according to the classification of World Health Organization (2010)) on the total of SUMO1-positive sperm. We found that 82.7±12.2% of SUMO1-ylated sperm were morphologically abnormal, whereas the percentage of SUMO1-ylated sperm with normal morphology was 17.3±12.2% ($P<0.00001$; Fig. 4A). Examples of SUMO1-ylated or not sumoylated sperm with apparently normal (white arrows) or abnormal morphology are shown in Fig. 4B. As can be observed, not only the majority of abnormal sperm show SUMO1-ylation, but in case of severe abnormality (such as in subject 3; Fig. 4B) intense staining is present, in agreement with Vigodner *et al.* (2013). Since the higher percentage of sumoylated sperm with abnormal morphology may be due to the high percentage of morphologically abnormal sperm of the patients (on average only 4.8±3.6% of sperm showed normal morphology in the 19 subjects), in order to confirm the relation between SUMO1-ylation and morphology, the same calculations were made on SUMO1-negative sperm. We found that a greater percentage of SUMO1 negative sperm were normal (56.6±36.9 vs 22.4±24.5% abnormal, $P<0.002$), thus confirming that morphologically normal sperm are less frequently SUMO1-ylated. Finally, we evaluated the percentage of sperm with normal and abnormal morphology on the total of sperm showing co-localisation between SUMO1 and DRP1, RanGAP1 and Topoisomerase IIα. We found that all the three proteins co-localise with SUMO1 in a higher percentage of morphologically abnormal sperm (Table 1).

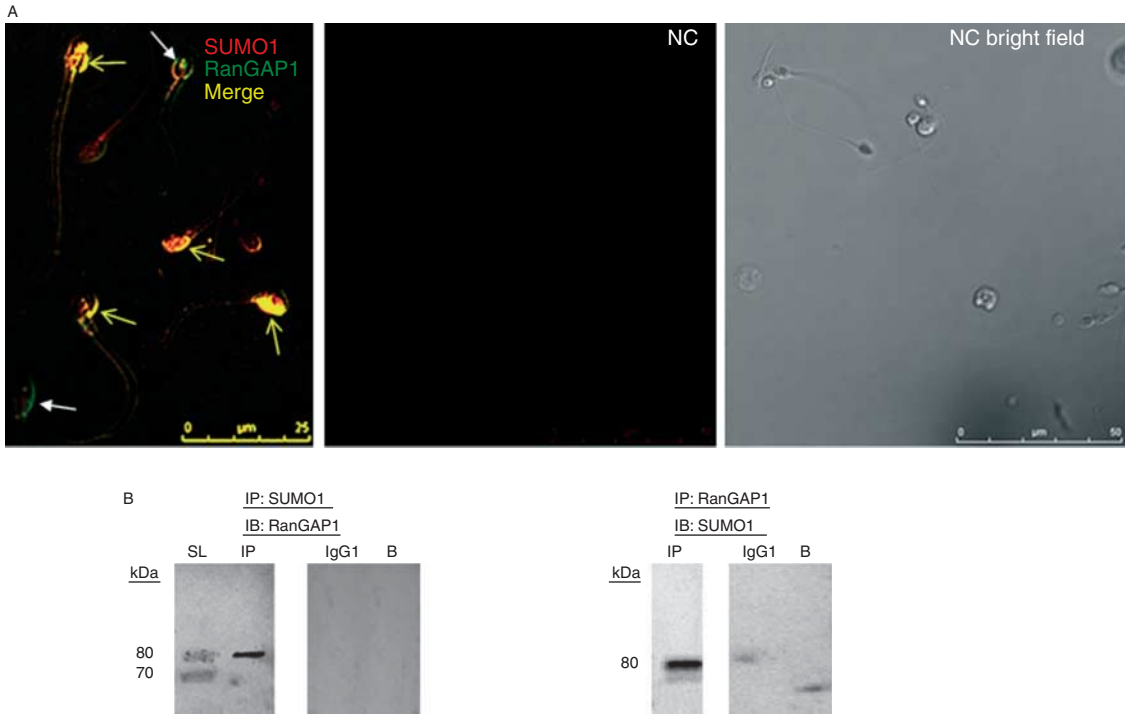

Figure 3 Identification of SUMO1-ylated RanGAP1 in human sperm. (A) Images obtained by immunofluorescence confocal laser microscopy of RanGAP1 (green signal, white arrows) and SUMO1 (red signal). RanGap1-positive spermatozoa were selected to compose the image. Yellow signal indicates co-localisation of the two proteins (yellow arrows) in the head. Negative control (NC) and the relative bright field (NC bright field) are shown in middle and right panels. (B, left panel) Western blot analysis of 40% PureSperm-selected sperm lysates (SL) and after immunoprecipitation with anti-SUMO1 antibody (IP:SUMO1), blotted with anti-RanGAP1 antibody. (B, right panel) Immunoprecipitated SL with anti-RanGAP1 antibody (IP:RanGAP1), blotted with anti-SUMO1 antibody. Negative controls have been performed using both only beads (B) and mouse IgG1 (IgG1). Images are representative of two similar experiments.

In the study by Vigodner *et al.* (2013), high levels of sumoylation were observed using both anti-SUMO1 and anti-SUMO2/3 antibodies, which showed similar patterns of expression and localisation in human sperm. Although our study was primarily focused on SUMO1-ylation, we also performed western blotting analysis of SL and immunofluorescence experiments with an anti-SUMO2/3 antibody to evaluate the pattern of SUMO2/3 expression in sperm. As can be observed in Fig. 5A, anti-SUMO2/3 antibody revealed a similar pattern of protein bands after being analysed by western blotting, with two major bands at about 80 and 60 kDa (Marchiani *et al.* 2011). Moreover, similar to anti-SUMO1 (Fig. 4B), immunofluorescence

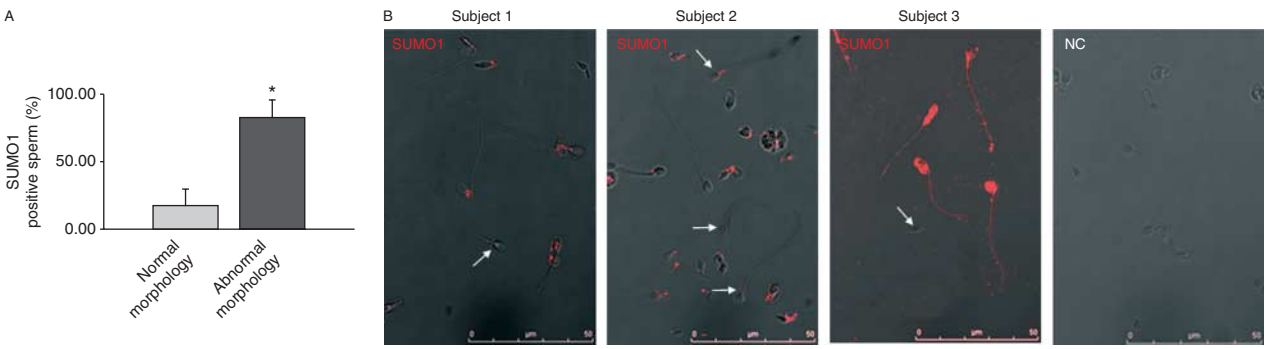

Figure 4 SUMO1 in morphologically normal and abnormal sperm. (A) Histograms reporting mean (±S.D.) percentage of sperm showing normal or abnormal morphology on the total of SUMO1 positive ($n=19$). *$P<0.00001$ vs normal morphology. (B) Images obtained by immunofluorescence confocal laser microscopy where SUMO1 signal (red fluorescence) and bright field are merged. Images are representative of three different subjects. Negative control (NC, right panel) is also shown. White arrows indicate apparently morphologically normal sperm.

Table 1 Co-localisation of DRP1, RanGAP1 and Topoisomerase IIα with SUMO1 in sperm with apparently normal and abnormal morphology.

	Normal morphology (%)	Abnormal morphology (%)	*P*	*n*
DRP1	20.7±17.7	78.5±16.6	<0.0001	13
RanGAP1	6.7±9.4	93.3±9.4	0.01	2
Topoisomerase IIα	18.8±23.9	81.3±23.9	0.01	4

The percentage of sperm with normal and abnormal morphology on the total of sperm showing co-localisation between SUMO1 and the three proteins are expressed as mean±S.D.

experiments with anti-SUMO2/3 antibody detected intense expression in morphologically abnormal sperm (Fig. 5B), in agreement with Vigodner *et al.* (2013).

Determination of the association between SUMO1-ylation and SDF

Recently, it has been demonstrated that SUMO and other components of the sumoylation machinery are often located at level of DSBs both in human somatic cells (Psakhye & Jentsch 2012) and mouse spermatocytes (Shrivastava *et al.* 2010), highlighting a role of such post-translational modification in the repair of DNA damage (Bekker-Jensen & Mailand 2011). To evaluate the possible association between the two parameters in sperm, we separately determined, by flow cytometry, SDF and SUMO1-ylation in the same ejaculates. SDF was evaluated by TUNEL/PI technique, a protocol that we have recently set up (Muratori *et al.* 2008, 2010), which allows coupling TUNEL assay with the use of the nuclear dye PI to perform cytometric analysis only in sperm population without other semen interferents. Moreover, such technique discriminates between two sperm population, namely $PI^{brighter}$ and PI^{dimmer}, which differ for several characteristics (Muratori *et al.* 2008). They show different staining for PI, different viability (PI^{dimmer} sperm are all dead, $PI^{brighter}$ are partly dead (Marchiani *et al.* 2011)) and different percentages of DNA-fragmented sperm (the percentage of SDF in $PI^{brighter}$ population is variable whereas PI^{dimmer} population is formed by 100% fragmented sperm (Muratori *et al.* 2008) and includes a fraction of sperm with large loss of chromatin material (Marchiani *et al.* 2014)). Most importantly, we have shown that although DNA-fragmented PI^{dimmer} sperm nicely negatively correlate with semen parameters, the percentage of SDF in $PI^{brighter}$ population does not (Muratori *et al.* 2008). As reported in our previous paper (Marchiani *et al.* 2011), we demonstrated that evaluation of SUMO1 positivity after a short-time (4 min) permeabilisation protocol mainly detects alive $PI^{brighter}$ sperm, the correlation analysis between SDF and SUMO1 has been conducted in the $PI^{brighter}$ population using the same short-time protocol. A positive correlation between the two parameters ($r=0.4$, $P=0.02$, $n=37$; Fig. 6A) was found, which suggests that SUMO1 and DNA fragmentation may be concomitantly present in sperm.

To study whether SDF and SUMO1 are concomitantly present in the same sperm, we stained the samples with three different fluorescences, marking SDF with TUNEL/FITC, SUMO1 with an AlexaFluor 546-conjugated secondary antibody and the nucleus with DAPI, which distinguish $PI^{brighter}$ and PI^{dimmer} sperm with the same efficiency as PI (unpublished results). As shown in Fig. 6B, which reports a typical experiment, after setting a gate to perform the analysis only in the $PI^{brighter}$ sperm (right upper panel) within the flame-shaped region characteristic of sperm (left upper panel), we calculated the percentage of $PI^{brighter}$ SUMO1-positive sperm that also resulted in DNA fragmentation (right lower panel, highlighted by grey rectangle). We found that 70.6±23.1% ($n=3$) of SUMO1-positive sperm (representing 11±6.8% of $PI^{brighter}$ sperm) were also DNA fragmented. Conversely, only 12.6±6.9% of DNA-fragmented $PI^{brighter}$ sperm showed concomitant SUMO1-ylation. In Fig. 6C, examples of sperm with both signals (SUMO1 and TUNEL, white arrows) are shown. In these sperm, the two signals mostly localised in the nucleus.

To further investigate the association between SUMO1 and SDF, the latter was induced by treatments (freezing and thawing and oxidative insult) known to increase it, and modifications of SUMO1 and SDF levels were evaluated thereafter. After freezing and thawing (Gavella *et al.* 2012, Meamar *et al.* 2012), the levels of both SDF (Fig. 7A) and SUMO1 (Fig. 7B) increased in the $PI^{brighter}$ population (% increase: 190.3±82.7, $P=0.002$ for SDF and 289.2±217.7, $P=0.02$ for SUMO, $n=6$). After such treatment, sperm viability decreased, on average, by about 25% (from 74.8±7.2 to 55.3±11.0%, $n=4$, $P=0.03$), confirming our previous data (Meamar *et al.* 2012). Western blotting analysis of

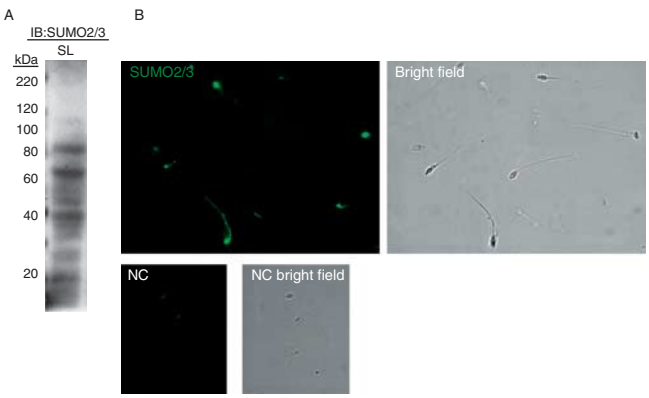

Figure 5 SUMO2/3 expression and localisation. (A) Western blotting analysis of 40% PureSperm-selected sperm lysates (SL) blotted with anti-SUMO2/3 antibody. Representative of two similar experiments. (B) Images obtained by fluorescence microscopy of SUMO2/3 (green signal) and corresponding bright field. Negative control (NC) and the relative bright field (NC bright field) are also shown.

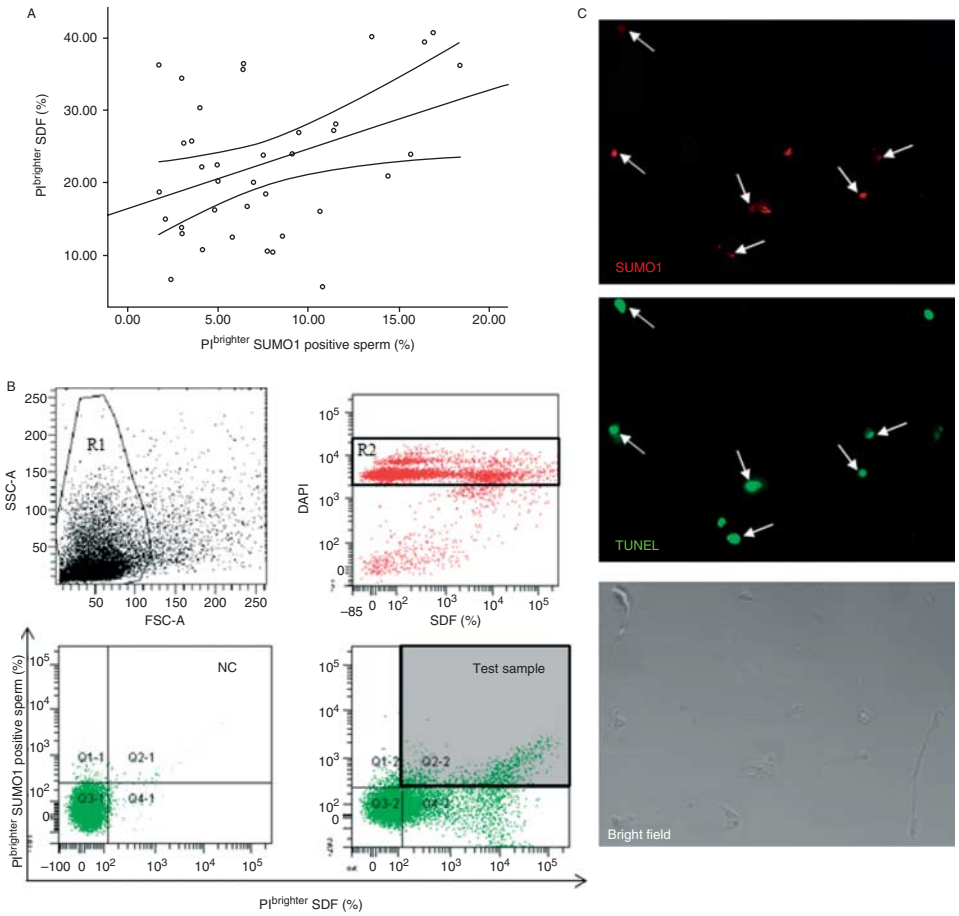

Figure 6 Association between SUMO1-ylation and SDF. (A) Correlation between the percentage of SDF and SUMO1-positive sperm calculated in the $PI^{brighter}$ population ($r=0.4$, $P=0.02$, $n=37$). (B) Typical experiment of triple labelling in order to detect simultaneously SDF (by TUNEL–FITC), SUMO1 (by using a secondary antibody conjugated with AlexaFluor 546) and DAPI (a nuclear dye used to discriminate $PI^{brighter}$ population) is shown. In left upper panel, the flame-shape region characteristic of sperm in FSC/SSC dot plot is shown (R1). Within R1, on the DAPI fluorescence dot plot (right upper panel), a gate on $PI^{brighter}$ sperm population is drawn (R2); in lower panels dot plots of SUMO1 and SDF fluorescences of negative control (NC, left) and test sample (right) within R2 are shown. The region of interest (Q2-2) with sperm showing co-presence of SUMO1 and SDF is highlighted by grey rectangle. Representative of three experiments. (C) Selection of sperm images made by immunofluorescence microscopy of TUNEL and SUMO1. Upper panel showed red signal of SUMO1, middle panel green signal of TUNEL and lower panel the corresponding bright field. White arrows indicate sperm where both SUMO1 and TUNEL localise in nucleus.

total SL after freezing and thawing demonstrated an increase in SUMO1-ylation of proteins (Fig. 7C). In particular, two protein bands, of about 75 and 100 kDa, showed higher SUMO1-ylation following the treatment (Fig. 7C, arrows).

After treatment with H_2O_2, 5 mM for 2 h (Smith *et al.* 2013), an increase in both SDF (% increase: 115.6 ± 72.2, $P=0.001$, $n=8$) and sperm sumoylation (% increase: 28.1 ± 28.2, $P=0.04$, $n=8$) was observed, albeit less pronounced with respect to freezing and thawing treatment.

As mentioned earlier, Topoisomerase IIα shows involvement in chromatin remodelling during spermiogenesis. In particular, the enzyme introduces DSBs, promoting the replacement of histones with protamines and, after completion of the process, is involved in repair of such breaks (McPherson & Longo 1993, Laberge & Boissonneault 2005). To investigate whether the increase in SDF and SUMO1 after freezing and thawing involves sumoylation of Topoisomerase IIα, we evaluated SUMO1-ylation of the protein by co-immunoprecipitation/western blotting analysis and co-localisation of the enzyme with SUMO1 by confocal microscopy after freezing and thawing procedure. As shown in Fig. 8B, an increase in Topoisomerase IIα SUMO1-ylation was observed. In addition, confocal microscopy experiments demonstrated increased co-localisation between Topoisomerase IIα and SUMO1 (Fig. 8C). In particular, evaluation of sperm percentage showing co-localisation of the two proteins on the total of Topoisomerase IIα positive revealed a significant increase in the percentage of sperm showing Topoisomerase IIα–SUMO1 co-localisation (32.9 ± 6.1 vs $50.7\pm10.6\%$, $P=0.04$, $n=4$) determined by freezing and thawing.

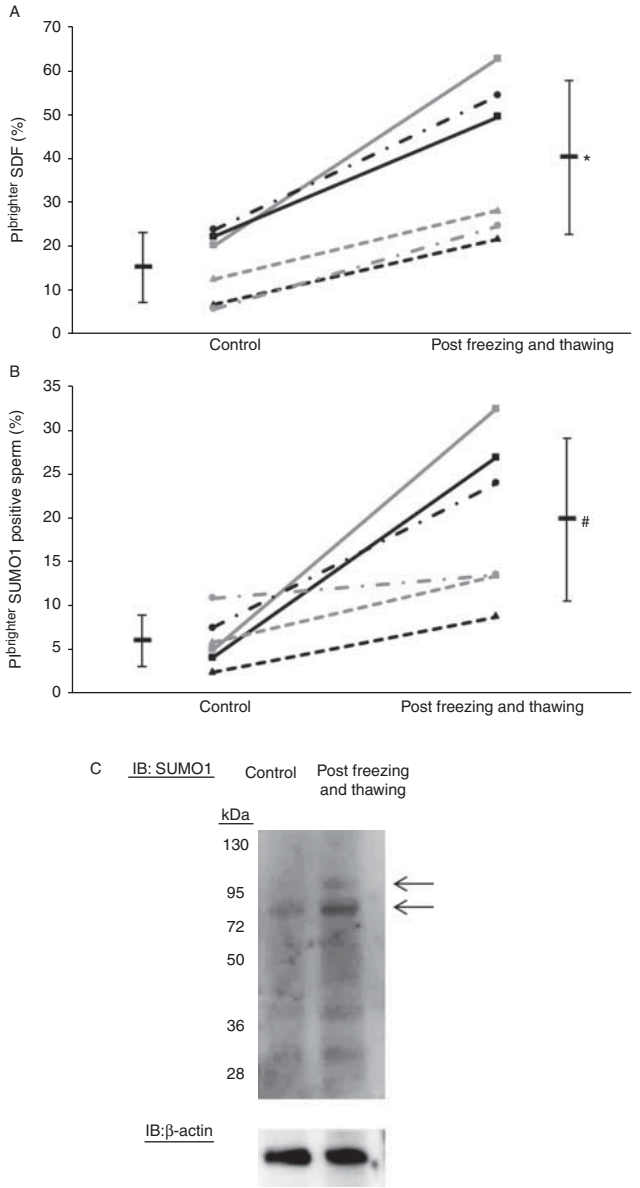

Figure 7 Sperm DNA fragmentation (SDF) and SUMO1-ylation after freezing and thawing. (A and B) Graphs show the increase in the percentage of SDF (A) and SUMO1 (B)-positive sperm after freezing and thawing of six samples. Means (±S.D.) are also included. $^{*}P=0.002$ (for SDF) and $^{\#}P=0.02$ (for SUMO1) vs Control. (C) Western blotting analysis of 40% PureSperm-selected pooled sperm lysate from control sperm and after freezing and thawing. The membrane was also blotted with anti-β-actin for loading control. The arrows indicate protein bands showing increased SUMO1 expression. Representative of two similar experiments.

Discussion

The present study identifies three SUMO1 targets in human sperm, namely DRP1, RanGAP1 and Topoisomerase IIα, and provides further insights about the relation between SUMO1-ylation and poor motility and morphology. In addition, we show evidence for an association between sperm SUMO1-ylation and SDF, and demonstrate that SUMO1-ylation may increase in mature sperm following stressful conditions being able to induce DNA fragmentation.

As mentioned earlier, previous studies investigating the role of SUMO pathways in human sperm highlighted an association between this post-translational protein modification and poor motility (Marchiani *et al.* 2011, Vigodner *et al.* 2013) and abnormal morphology (Vigodner *et al.* 2013), and such association is likely driven by the nature of sperm SUMO targets besides the extension of the process in the spermatozoon. In this study, we have investigated sumoylation of three possible SUMO targets. Among these, DRP1, a GTPase belonging to the family of dynamins involved in mitochondrial fission (Smirnova *et al.* 2001, Otera *et al.* 2013), was chosen in view of the fact that its sumoylation leads to an association with mitochondrial membranes of somatic cells (Harder *et al.* 2004) where, if sumoylation of the protein is stably maintained by silencing the de-sumoylating enzyme SENP5 (Zunino *et al.* 2007) or artificially increased by overexpressing SUMO1 (Harder *et al.* 2004), severely altering mitochondrial morphology and function. By western blot analysis, confocal and electron microscopy experiments we demonstrate in this study that DRP1 is expressed in ejaculated sperm and is located within the principal and

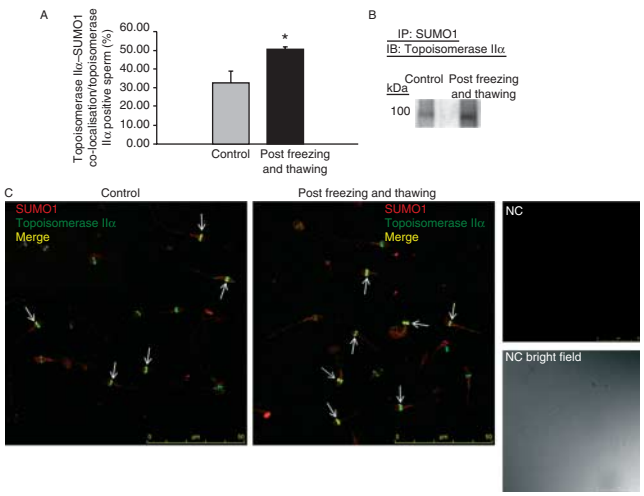

Figure 8 Topoisomerase IIα–SUMO1 co-localisation after freezing and thawing. (A) Histograms reporting mean (±S.D.) percentage of sperm showing Topoisomerase IIα–SUMO1 co-localisation on the total of Topoisomerase IIα-positive in three semen samples. $^{*}P=0.04$ vs Control. (B) Western blotting analysis of 40% PureSperm-selected pooled sperm lysates from control sperm and after freezing and thawing after immunoprecipitation with anti-SUMO1 (IP:SUMO1) antibody and blotting with anti-Topoisomerase IIα antibody. Representative of two similar experiments. (C) Images related with immunofluorescence confocal laser microscopy of Topoisomerase IIα (green signal) and SUMO1 (red signal) in control conditions and after freezing and thawing. Yellow signal indicates co-localisation of the two proteins (white arrows). Negative control (NC) and the relative bright field (NC bright field) are also reported. Images are representative of three different experiments.

end pieces of the tail. Such a location is not surprising in view of the fact that DRP1 has been found to associate with microtubules in somatic cells (Yoon *et al*. 1998, Strack *et al*. 2013). We also demonstrate that the protein is SUMO1-ylated and that co-localisation of SUMO1 and DRP1 mostly occurs at the mid-piece level, where mitochondria are located. Interestingly, we find that co-localisation of DRP1 with SUMO1 is higher in the sperm of asthenozoospermic subjects compared with normospermic, and the percentage of sperm showing such co-localisation negatively correlated with the percentage of motility of the samples, suggesting a link between SUMO1-ylation of DRP1 and poor sperm motility. We hypothesise that DRP1 localises to the mitochondria following sumoylation, leading to alterations of the organelles and consequent impairment of motility, if sumoylation persists (Zunino *et al*. 2007). Further studies are, however, necessary to better understand the role of DRP1 sumoylation/de-sumoylation in motility. In addition to DRP1, other glycolytic and mitochondrial enzymes have been demonstrated to be SUMO2/3 targets in sperm (Vigodner *et al*. 2013). Although the role of SUMO2/3-ylation of these mitochondrial proteins is unknown, a scenario configures where several SUMO-modified proteins may lead to reduced sperm motility through alterations in mitochondrial functions or other metabolic processes that provide energy (referred also in Vigodner *et al*. (2013)).

Another SUMO1-ylated protein in ejaculated sperm is Topoisomerase IIα. This protein plays a key role during chromatin condensation in the last phases of spermatogenesis. Indeed, it introduces DNA strand breaks to favour transition of histone to protamine and, later on, is involved in DNA re-ligation (Laberge & Boissonneault 2005). This protein is present in the post-acrosomal region of the sperm head, compatible with localisation in the nuclei (Har-Vardi *et al*. 2007), where an intense SUMO1 signal is present (Marchiani *et al*. 2011). Previous studies demonstrated that Topoisomerase IIα is SUMO2/3-ylated in germ cells during meiosis, localising to centromeric heterochromatin (Shrivastava *et al*. 2010), where it may have a role in sister chromatid separation during cell division as it occurs in somatic cells (Lee & Bachant 2009) and in *Xenopus* oocytes (Azuma 2009). Thus, sumoylation of Topoisomerase IIα in mature sperm may represent a remnant of spermatogenesis persisting in immature or morphologically altered sperm. The lack of a trend to correlation between the percentage of spermatozoa showing co-localisation between SUMO1 and Topoisomerase IIα and sperm motility rules out a role in motility regulation and reinforces the hypothesis that the role of sumoylation in sperm depends on the nature and localisation of the targets. The exclusive nuclear localisation of the protein (Fig. 2) is not compatible for a possible role in sperm motility. However, in view of our results showing that SUMO1-ylation of Topoisomerase IIα increases following stressful conditions, we cannot exclude the possibility that sumoylation of this protein may play an active role in sperm functions (see later on for further discussion about this point).

In this study, we have demonstrated that RanGAP1, another protein with GTPase activity as DRP1, is SUMO1-ylated in human sperm where it is localised in the post-acrosomal region of the sperm head. In somatic cells, RanGAP1 acts as a key controller of nucleocytoplasmic trafficking processes and is required for other functions such as spindle assembly and cell cycle control by catalysing the hydrolysis of GTP to GDP in RanGTPase (Lui & Huang 2009). SUMO1-ylation of RanGAP1 allows targeting cytoplasmic filaments of the nuclear pore complex (Matunis *et al*. 1998). Moreover, Ran GAP1/RanGTPase complex seems to be involved in the organisation of structures containing microtubules and has been found to localise to the manchette and the centrosome of spermatids where it may be involved in the control of traffic of nuclear proteins during chromatin condensation and in tail development (Dasso 2001, Kierszenbaum *et al*. 2002). Thus, it is possible that sumoylation of RanGAP1 occurs during spermatogenesis, as also recently hypothesised based on the occurrence of a main 85 kDa SUMO1-ylated band in testicular cells (Shrivastava *et al*. 2010). The finding that SUMO1-ylation of RanGAP1 is associated with morphologically abnormal sperm suggests that de-sumoylation of the protein should normally occur. However, we cannot exclude that sumoylated RanGAP1 may have other roles in mature sperm in protein trafficking between the nucleus and the cytoplasm or in the fertilisation process.

Overall, our study confirms and extends previous data showing an association between SUMO and poor sperm motility and morphology further supporting the notion that sumoylation marks defective sperm (Marchiani *et al*. 2011, Vigodner *et al*. 2013). These results suggest that protein sumoylation, likely necessary during spermatogenesis and spermiogenesis (Vigodner & Morris 2005, Vigodner *et al*. 2006, Vigodner 2011), should be mostly eliminated. However, owing to the fact also that spermatozoa with apparent normal morphology (Vigodner *et al*. 2013 and present study) may be sumoylated (although, likely, at lower extent) by other functions which cannot be excluded. One of such functions may be related with DNA integrity. In somatic cells, protein sumoylation has been associated with increased DNA stability (Bekker-Jensen & Mailand 2011), although how DNA repair mechanisms are affected by SUMO modifications of proteins is poorly understood. In this study, to evaluate the association between SDF and sumoylation, we have used a protocol that allows the detection of both parameters in $PI^{brighter}$ population (Muratori *et al*. 2008, Marchiani *et al*. 2011). $PI^{brighter}$ population is of high interest for the fertilisation process, as, although it may show DNA fragmentation, the latter is not related with semen quality (Muratori *et al*.

2008), implying that a $PI^{brighter}$ sperm with damage in its DNA may be motile and/or morphologically normal. Moreover, we have previously shown that, with such a protocol, the great majority of sumoylated $PI^{brighter}$ sperm are alive (Marchiani *et al*. 2011). The occurrence of a significance correlation between SDF and sumoylation and, most importantly, the demonstration that most SUMO1-ylated sperm, in a given sample, is concomitantly DNA fragmented, indicate that SUMO1, when evaluated using a short-time permeabilisation protocol, might identify a fraction of live DNA-fragmented sperm in $PI^{brighter}$ population. Whether the simultaneous occurrence of the two parameters in the same sperm is related with induction of the damage, an attempt to repair it, or is simply casual is presently unknown. However, the occurrence of $PI^{brighter}$ sperm with co-localisation of the two signals in the nucleus and the demonstration that when DNA fragmentation is induced in mature sperm by freezing and thawing or oxidative insults, also sumoylation increases (Fig. 7), as it occurs in somatic and germ cells (Lee & Bachant 2009, Shrivastava *et al*. 2010), suggests a possible active involvement of this post-translational modification in responses to stressful conditions. It is possible that the increase in sumoylation occurs in an attempt to repair or to limit the damage to DNA. In somatic cells, when SUMO pathways are inhibited, the apoptotic damage in response to genotoxic stimuli increases (Mo *et al*. 2004, Wu *et al*. 2007), indicating that SUMO is involved in attenuating it.

Overall, our results that showed an increase of sumoylation after freezing and thawing and oxidative insult indicate that sumoylation pathways are present in mature sperm and can be activated. Although we cannot exclude that the membrane damage, provoked by freezing and thawing procedure, allows a better penetration of the anti-SUMO1 antibody leading to an increase in SUMO1-ylated sperm, the demonstration by western blotting analysis of an increase in SUMO1-ylated protein bands after the procedure and the increased Topoisomerase IIα SUMO1-ylation in immunoprecipitation/western blotting experiments, indicate that, at least in part, the increase in the percentage of SUMO1-ylated sperm is due to activation of the pathway. Moreover, the finding that Topoisomerase IIα–SUMO1 co-localisation increases following freezing and thawing procedure argues against an increased penetration of the anti-SUMO1 antibody, as a long-time permeabilisation protocol (allowing to unmask all sumoylation present) has been used in these experiments. We cannot exclude that freezing and thawing and oxidative insult increase sumoylation through inhibition of de-sumoylation pathways, as it occurs in somatic cells following heavy stress stimuli (Xu *et al*. 2008). At present, however, occurrence and understanding the role of de-sumoylating enzymes in human sperm require further studies.

The increase in Topoisomerase IIα SUMO1-ylation in sperm following freezing and thawing is not surprising as stressful conditions have been shown to increase the sumoylation of topoisomerases in somatic cells (Lee & Bachant 2009). It should be noted that Topoisomerases II have been shown to express unique properties in sperm, retaining DNA relaxation activity and lacking the decatenation one (Har-Vardi *et al*. 2007). However, which could be the role of these modified enzymes in mature sperm is still questioned (Har-Vardi *et al*. 2007). Topoisomerase IIβ has been shown to cleave DNA in an apoptotic-like manner in mouse sperm (Shaman *et al*. 2006), whereas the addition of Topoisomerase II inhibitors to the fertilisation medium leads to the occurrence of DNA breaks in decondensing sperm (Bizzaro *et al*. 2000). Thus, sperm Topoisomerases II may be involved both in generating and repairing DNA damage.

In conclusion, our study identified DRP1, RanGAP1 and Topoisomerase IIα as targets of SUMO1 in human-ejaculated sperm, giving more insights on the role of SUMO1-ylation in sperm morphology and motility. In addition, we show an association between SUMO1-ylation and SDF and demonstrate that, when evaluated using a short-time permeabilisation protocol, SUMO1 co-localises with TUNEL in $PI^{brighter}$ sperm. The demonstration that the percentage of sumoylated sperm increases in mature sperm following stressful conditions in concomitance with SDF highlights a possible active role of this post-translational modification following ejaculation.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-14-0173.

Declaration of interest

S Marchiani, L Tamburrino, B Ricci, D Nosi, M Cambi, P Piomboni, G Belmonte, G Forti, M Muratori and E Baldi declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research.

Funding

This work was supported by grants from Italian Ministry of University and Scientific Research (project FIRB to S Marchiani and PRIN to E Baldi) and Regione Toscana (to G Forti).

Acknowledgements

The authors thank Prof. Mario Maggi for helpful advice, and Drs Erminio Filimberti, Selene Degl'Innocenti and Maria Grazia Fino (AOUC Careggi) for semen analysis.

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Received 31 March 2014
First decision 6 May 2014
Revised manuscript received 9 July 2014
Accepted 7 August 2014