Human Reproduction, Vol.26, No.9 pp. 2274–2282, 2011
Advanced Access publication on July 19, 2011 doi:10.1093/humrep/der232

human reproduction

ORIGINAL ARTICLE *Andrology*

Light-mediated activation reveals a key role for protein kinase A and sarcoma protein kinase in the development of sperm hyper-activated motility

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Submitted on November 19, 2010; resubmitted on June 12, 2011; accepted on June 20, 2011

BACKGROUND: Hyper-activated motility (HAM) is part of the sperm capacitation process, which is necessary for fertilization. In this study, we investigated the effect of visible light on sperm motility and hyperactivation and evaluated pathways mediating these effects.

METHODS: Human sperm (1×10^7 cells/ml) in capacitation media were irradiated for 3 min with 40 mW/cm^2 visible light (400–800 nm with maximum energy at 600 nm). Sperm motility was assessed and analyzed by computer-assisted sperm analysis. The involvement of sperm capacitation factors was investigated as follows. The generation of reactive oxygen species (ROS) was measured using 20,70-dichlorofluorescein diacetate. Protein kinase A (PKA) and sarcoma protein kinase (Src) activity were measured using western blot analysis and inhibited using 50 μM H89 and 10 μM PP2, respectively. Soluble adenlyl cyclase was inhibited using 20 μM 2-OH-Estradiol. The intracellular concentration of free Ca^{2+} was assessed using the fluorescent calcium indicator, Fluo-4/AM. Sperm DNA fragmentation was determined using the sperm chromatin dispersion test.

RESULTS: Light irradiation of human sperm caused a significant increase in hyper-HAM but not total motility. The production of ROS and activation of soluble adenylyl cyclase and PKA mediated the effect of light on HAM. Light irradiation also activated Src, and inhibition of Src significantly reduced the effect of light on HAM. Light irradiation caused a rapid increase in intracellular Ca^{2+} concentration and the increase in HAM was significantly reduced when voltage-dependent-Ca^{2+}-channel activity was blocked or when Ca^{2+}-deficient medium was used.

CONCLUSIONS: Light irradiation of human sperm for a short time causes a significant increase in HAM in a mechanism mediated by ROS production, activation of PKA, Src and Ca^{2+} influx.

Key words: sperm / capacitation / light / hyper-activated motility / protein kinase A

Introduction

Light affects biological cells; however, the resulting biochemical cascade following irradiation is not completely understood. Previous studies have shown that light irradiation at different wavelengths can increase cell proliferation (Grossman *et al.*, 1998; Kreisler *et al.*, 2002), enhance diabetic wound-healing (Forney and Mauro, 1999) and increase motility and fertilization rates in sperm cells (Cohen *et al.*, 1998). Laser irradiation has also been found to influence the activity of some NADH-linked mitochondrial reactions in isolated liver mitochondria *in vitro* (Passarella *et al.*, 1982, 1983), and He–Ne laser irradiation was shown to generate mitochondrial extra electrochemical potential as well as to cause an increase in ATP synthesis (Passarella *et al.*, 1984). The photobiostimulation process occurs when porphyrins, flavins or cytochromes absorb light and transfer the absorbed energy to oxygen molecules around them, creating reactive oxygen species (ROS). Relatively low concentrations of ROS are known to mediate various intracellular processes, and in sperm cells ROS have a pivotal role in cellular physiology and fertilization capability (de Lamirande *et al.*, 1997; Leclerc *et al.*, 1997). It is well accepted that sperm must undergo several biochemical changes called 'capacitation' in order to fertilize. It is known that sperm develop hyper-activated motility (HAM) during the capacitation process (Burkman, 1984; Mortimer and Mortimer, 1990), which is characterized by an increase in flagellar bending amplitude, and an

increase in average lateral head movement (Mortimer and Mortimer, 1990). It was shown that hyper-activated sperm penetrate the zona pellucida much more effectively than non-hyper-activated sperm (Stauss *et al*., 1995); so it has a great importance in the fertilization process. Superoxide anion and hydrogen peroxide both play a role in capacitation of human sperm (Griveau and Le Lannou, 1997) and have a positive effect on HAM and protein tyrosine phosphorylation (de Lamirande and Gagnon, 1993; Griveau *et al*., 1994; Leclerc and Gagnon, 1996). Human spermatozoa incubated under capacitation conditions produce more superoxide anion than control cells (Aitken and Vernet, 1998). In addition, low concentrations of hydrogen peroxide elevate protein tyrosine phosphorylation during capacitation (Aitken and Vernet, 1998) and induce protein kinase A (PKA) activity (Rivlin *et al*., 2004). Additionally, we found that light irradiation enhances the *in vitro* fertilization capability of mouse sperm (Cohen *et al*., 1998).

In the current study, we examined the effect of visible light irradiation on human sperm HAM. Light irradiation of non-capacitated sperm for 3 min elevated both ROS production and HAM. Examining the mechanism of the effect of light on hyperactivation revealed that PKA, Src and Ca^{2+} influx activities mediated this effect.

Materials and Methods

Media and antibodies

Capacitation medium, F-10 (HAM) nutrient mixture with L-Glutamine, was purchased from Biological Industries (Kibbutz Beit Haemek, IL). Antibodies against phospho-PKA substrate (RRXS/T) (100G7) and phospho-Src family (Tyr416) (100F9) were purchased from Cell Signaling (Beverly, MA, USA). Goat Anti-Rabbit IgG (H+L)-HRP conjugate and Goat Anti-Mouse IgG (H+L)-HRP conjugate were obtained from Bio-Rad (BioRad Lab., Richmond, CA, USA). All other chemicals were purchased from Sigma (Sigma-Aldrich Israel Ltd., Rehovot, Israel), unless otherwise stated.

Sperm preparation

Human semen was liquefied, loaded on a Percoll gradient (80, 40 and 20%) and centrifuged for 30 min at 690*g* at room temperature. The lower layer containing the sperm was collected and washed twice in HAM F-10, then spun again and allowed to 'swim-up' after the last wash at 37°C. The motile cells were collected without the pellet and resuspended in capacitation medium. This procedure allows us to obtain motile sperm without contamination of leukocytes.

Exposure of sperm to light

Human sperm (1×10^7 cells/ml) were placed in capacitation media [HAM F-10 supplemented with 3 mg/ml bovine serum albumin (BSA)]. Samples of 2 ml were placed in a 35 mm tissue culture dish and irradiated for 3 min with 40 mW/cm^2 visible light (400–800 nm) with maximum energy at 600 nm. This light intensity is like the midday sunlight without the emission of UV or IR light.

Sperm capacitation

Human sperm (1×10^7 cells/ml) were capacitated by incubation in capacitation media, HAM F-10 supplemented with 3 mg/ml BSA. The cells were incubated in this capacitation medium for 3 h at 37°C in 5% CO_2. The capacitation state of the sperm was confirmed after 3 h incubation by examining the ability of the sperm to undergo the acrosome reaction.

Sperm motility determination

Sperm cells (1×10^7 cells/ml) were incubated for capacitation in HAM F-10 + BSA medium. Samples (5 μl) were placed in prewarmed standard count four chamber 20 μm-depth slide (Leja, Nieuw-Vennet, Netherlands) at 37°C, and analyzed using a computer-assisted sperm analysis (CASA) device with IVOS software (version 12, Hamilton-Thorne Biosciences). Up to 10 sequences, each 30 s long, were acquired for each sample. At least 100 cells were analyzed in each sample, according to parameters identifying human sperm motility. The proportion of hyper-activated spermatozoa in each sample was determined using the SORT function of the CASA instrument. HAM was defined by curvilinear velocity (VCL) >100 μm/s and linearity (LIN) <60% and amplitude of lateral head (ALH) >5 μm (Mortimer and Mortimer, 1990).

ROS measurement

Measurement of ROS production was used for the assessment of intracellular ROS. Cells (2.5×10^5 cells/ml) were placed in a black 96 well plate. The generation of ROS was measured using 20,70-dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich Israel Ltd), which is incorporated into the cells and cleaved into fluorescent DCF in the presence of ROS. For this assay, 10 μM DCFDA was added to the cell suspension in a black plate. The cells were incubated in the dark at 37°C for 30 min. Cells were irradiated for 3 min after incubation. Superoxide dismutase (SOD) and the mitochondrial respiration inhibitor, sodium azide (60 units and 10 μM, respectively) were used to confirm that most of the increase of fluorescence is due to ROS production by the cells and only a small increase is due to photosensitization of DCF. DCF fluorescence was measured by Tecan spectrofluorometer microplate reader using 485 nm for emission and 535 nm for excitation.

ROS detection by microscope

Cells were incubated with 30 μM DCFDA for 30 min in the dark. The loaded cells were washed to remove extracellular DCFDA, then 10 μM sodium azide was added to some samples and 5 min later cells were irradiated for 3 min, and placed on a slide and covered with cover glass. Images were captured in Olympus AX70 microscope equipped with Olympus DP50 digital camera and by 'Viewfinder Lite' software (version 1 from Pixera Corporation), at a magnification of ×400. All cell preparations from a single experiment were photographed in the same session and for the same exposure period.

Immunoblot analysis

Sperm cells were washed by centrifugation for 5 min at 10 000*g* at 4°C, and then the supernatant was discarded, and the pellet was resuspended in Tris-buggered saline (TBS) and centrifuged again in order to remove remaining BSA. Sperm preparations were then lysed by the addition of lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 6% SDS, protease inhibitor cocktail 1:100, 50 mM NaF, 50 mM pyrophosphate, 0.2 mM Na_3VO_4 and freshly added 1 mM phenylmethylsulfonyl fluoride to the pellet, and the lysate was vortexed vigorously for 15 min at room temperature. Lysates were then centrifuged for 5 min at 10 000*g* at 4°C, the supernatant was transferred and the protein concentration was determined by the Bradford method (Bradford, 1976) or by the BCA method (Smith *et al*., 1985). Sample buffer was added to the supernatant and boiled for 5 min. The extracts were separated on 10% SDS-polyacrylamide gels, and then electrophoretically transferred to nitrocellulose membranes.

Blots were routinely stained with Ponceau solution to confirm equal loading and even transfer. The blots were blocked with 1% BSA in TBS, pH 7.6, containing 0.1% Tween 20 (TBST), for 30 min at room temperature. Proteins were immunodetected using anti-phospho-PKA substrate,

phospho-Src or anti-tubulin (loading control) (Sigma), diluted 1:10 000. The membranes were incubated overnight at 4°C with the primary antibodies diluted in 1% BSA in TBST. Next, the membranes were washed three times with TBST and incubated for 1 h at room temperature with specific HRP-linked secondary antibodies (Bio-Rad Lab), diluted 1:5000 in TBST and 1% BSA. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence (Amersham, Little Chalfont, UK).

For re-blotting with an additional antibody, the membrane was stripped for 30 min at room temperature with gentle agitation using Re-Blot Plus Strong Solution, then blocked twice with 1% BSA in TBST for 30 min at room temperature and subjected to another antibody.

Determination of intracellular calcium

The intracellular concentration of free Ca^{2+} was assessed using the fluorescent calcium indicator, Fluo-4/AM. Washed cells (1×10^7/ml) were incubated with 1 μM Fluo-4/AM for 1 h. The loaded cells were then washed three times to remove extracellular Fluo-4/AM. The cells were resuspended in a transparent medium containing calcium and placed in a black 96 plate. Cells were irradiated for three minutes, and fluorescence was immediately measured for a period of 10 min using Tecan spectrofluorometer microplate reader, with an excitation wavelength of 488 nm and emission of 516 nm.

Assessment of DNA fragmentation

DNA fragmentation was assayed with Halosperm kit based on the sperm chromatin dispersion (SCD) test (Fernandez *et al.*, 2003, 2005). The method was performed according to the manufacturer's directions.

Statistical analysis

Data are expressed as mean ± SD or SE of at least three experiments for all determinations. Statistical significance was calculated by student's *t*-test or by analysis of variance with 'Bonferroni's *post hoc* comparison test using SPSS software (Chicago, IL, USA).

Results

Effect of light on sperm motility

It was previously shown that light irradiation of various mammalian sperm enhances their motility (Sato *et al.*, 1984; Lenzi *et al.*, 1989; Corral-Baques *et al.*, 2005; Zan-Bar *et al.*, 2005). We showed elsewhere that irradiation of mouse sperm using a low power laser at 640 nm causes a significant increase in intracellular calcium and about 40% enhancement of the *in vitro* fertilization rate (Cohen *et al.*, 1998). It is well established that intracellular calcium plays a pivotal role in sperm motility regulation (Morton *et al.*, 1979); we therefore wished to determine if irradiation of human sperm would similarly affect their motility. Figure 1B shows the irradiation of human sperm incubated in capacitation medium by exposure to visible light (400–800 nm) for 3 min at 40 mW/cm^2, caused more than a 100% increase in HAM (Fig. 1B). However, no effect was seen on total motility (Fig. 1A). The enhanced effect of light on HAM was seen within 10 min of incubation and it continued for at least 3 h (Fig. 1B). Based on these results, we wished to understand the mechanisms by which light affects sperm motility.

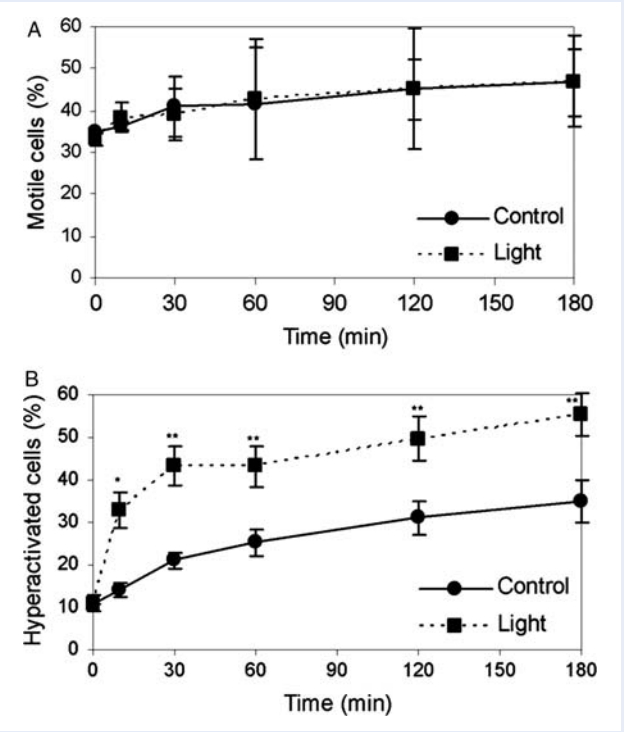

Figure 1 Effect of light on sperm motility. Sperm cells from 30 donors were irradiated with visible light for 3 min at 40 mW/cm^2. Motility was measured at different times during capacitation in CASA. (**A**) Effect of light on total motility during capacitation. (**B**) Effect of light on HAM during capacitation. Each graph represents the average of 30 different experiments ± SE at each time point. *$P < 0.05$, **$P < 0.001$.

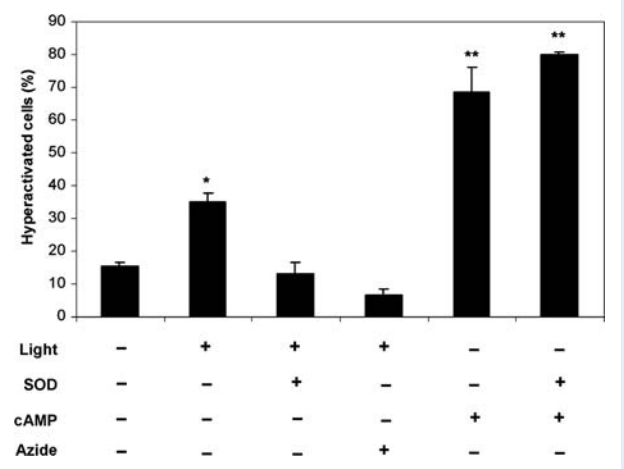

Figure 2 Involvement of ROS in HAM development. Sperm cells were incubated with 60 U of SOD, 10 μM sodium azide and/or 1 mM 8-Br-cAMP, for 10 min, and then irradiated for 3 min. HAM was measured 5 min after irradiation using CASA. The graph represents the average of three different experiments ± SD at each time point. *Significance compared with control $P < 0.05$. **$P < 0.01$.

Involvement of ROS

It is known from many studies that light affects biological cells via ROS production (Karu, 1989; Callaghan *et al.*, 1996; de Lamirande *et al.*, 1997; Cohen *et al.*, 1998; Oren *et al.*, 2001; Lavi *et al.*, 2003; Lubart *et al.*, 2005). One of these ROS is superoxide anion, which can be converted to hydrogen peroxide by SOD. Addition of SOD to the sperm suspension before light irradiation prevented the increase in HAM after light exposure (Fig. 2). Moreover, SOD prevented the development of HAM during sperm capacitation in the control cells, but not in the presence of 8Br-cAMP (Fig. 2) indicating that ROS might enhance cAMP production as discussed later. These data suggest that ROS are produced during the incubation, and ROS production is enhanced by light irradiation. Indeed our data revealed that ROS were produced after 1–3 min of light irradiation, while there was a significant reduction of ROS in the presence of SOD or the mitochondrial respiration inhibitor, azide (Fig. 3A). Although the washed sperm do not contain any detected leukocytes, it is possible that ROS is produced by the sperm and by contamination of leukocytes (reviewed by Ford, 2004). It was clearly shown that light significantly enhances ROS production (Fig. 3B). The significant inhibition in ROS production by azide (Fig. 3A and B) and enhanced fluorescence by light in the sperm midpiece, the location of the mitochondria (Fig. 3B), clearly indicated that the sperm mitochondrial respiration is the main source of ROS produced by light illumination. Also, HAM in DCF loaded cells was enhanced by light as in the control cells (not shown). Azide also caused a complete inhibition of the light effect on HAM (Fig. 2), indicating that HAM is driven by ROS produced by mitochondria, most likely sperm mitochondria.

Visible light irradiation did not affect DNA integrity, as measured by the SCD test (Fig. 4).

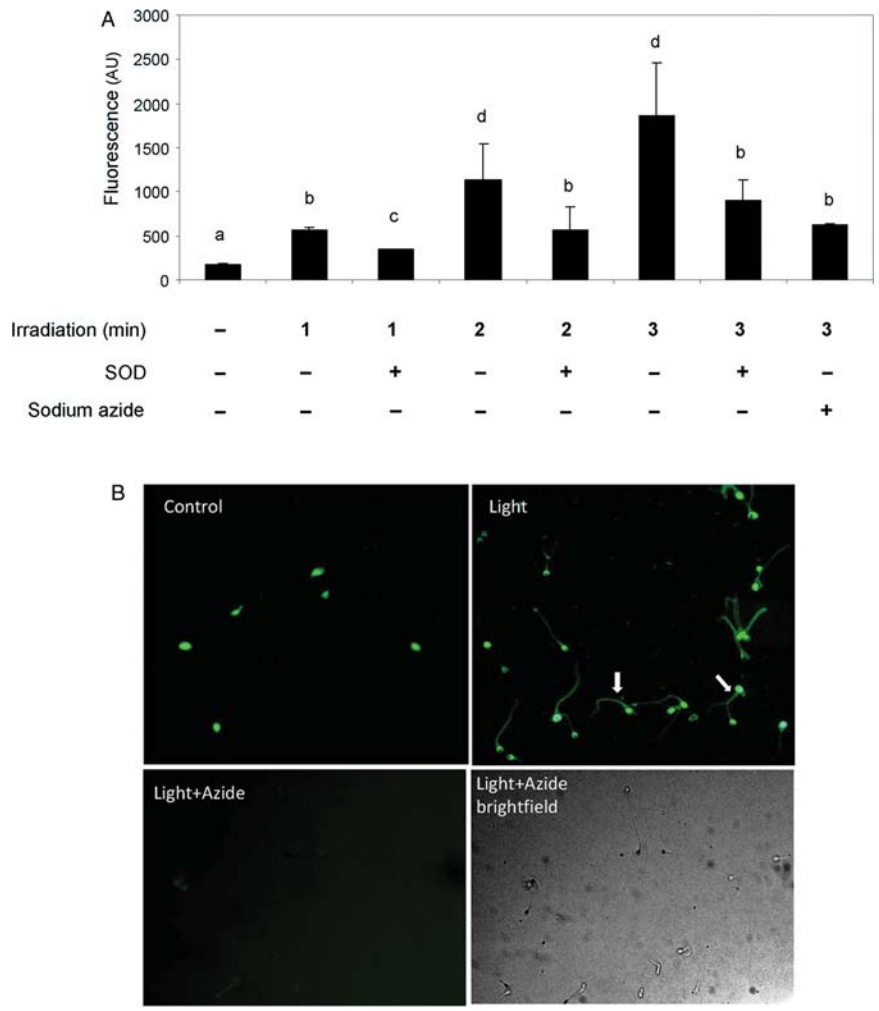

Figure 3 ROS formation in irradiated cells. (**A**) Cells were incubated with 10 μM DCFDA for 30 min. SOD at a concentration of 60 U or 10 μM Sodium azide were added to some samples and cells were irradiated for different times. Cells were placed in a black plate, and fluorescence was measured at 485/535 nm. The graph represents the average of three independent experiments ± SD at each time point. Different letters indicate significance using Duncan test ($P < 0.05$). (**B**) Cells were incubated with 30 μM DCFDA for 30 min 10 μM Azide was added to some samples and cells were irradiated for 3 min. Fluorescence indicating ROS in irradiated cells is viewed in the midpiece (arrows) and is absent in the presence of the respiration inhibitor, azide.

Involvement of cAMP dependent PKA

In sperm cells, about 95% of the adenylyl-cyclase consists of the bicarbonate-dependent soluble adenylyl-cyclase (sAC) isoform (Chen et al., 2000). sAC/PKA-dependent protein tyrosine phosphorylation occurs during sperm capacitation (Aitken et al., 1998; Visconti and Kopf, 1998; Visconti et al., 1999; Lefievre et al., 2002). However, it is not clear whether the development of HAM depends on PKA. In bovine sperm, it was suggested that HAM is PKA-independent (Marquez and Suarez, 2004); however, in mouse and boar sperm HAM development depends on PKA activity (Nolan et al., 2004; Baker et al., 2006; Harayama and Nakamura, 2008; Kaneto et al., 2008). Figure 5A shows that 3 min light irradiation of human sperm enhanced PKA activity within 3 min; PKA levels were further increased up to 3 h of incubation under capacitation conditions. This brief light irradiation (3 min) caused a significant increase in HAM, which was completely blocked by the PKA inhibitor, H89 (Fig. 5B). Moreover, HAM did not develop in the absence of bicarbonate in the incubation medium, with or without light treatment (Fig. 6), though HAM could be recovered by adding the permeable 8Br-cAMP to the incubation medium (Fig. 6). These data suggest that sAC activity is essential for cAMP production needed for PKA activation. This suggestion is further supported by the complete inhibition of light-induced HAM by 2-OH-estradiol, a specific inhibitor of sAC (Fig. 6).

It has been suggested elsewhere that PKA can activate the Src tyrosine kinase in sperm cells (Baker et al., 2006). We showed recently in bovine sperm that PKA can activate Src, leading to the activation of the epidermal growth factor receptor (EGFR) (Etkovitz et al., 2009). Light treatment of human sperm enhanced the Src phosphorylation/activation on Tyr416 (Fig. 7A). Moreover, the development of HAM in human sperm was completely blocked by PP2, a specific inhibitor of SRC-family kinases (Fig. 7B). However, the development of HAM in the control cells or in the light-treated cells was not affected by PP3 (Fig. 7B), an inactive analog of PP2, indicating the specificity of PP2 as a Src-family inhibitor.

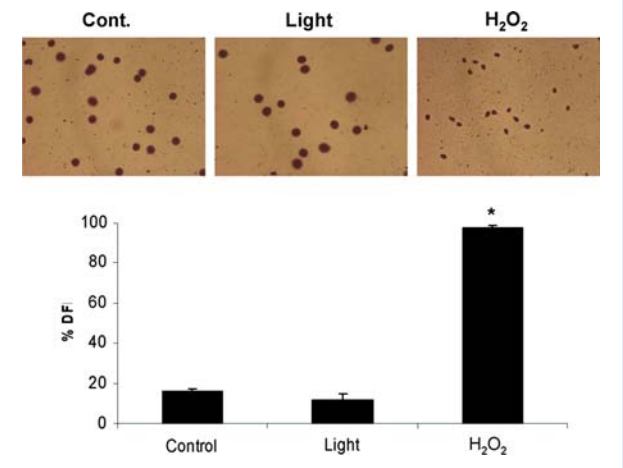

Figure 4 Sperm DNA fragmentation determined using the SCD test. Samples were irradiated for 3 min or incubated with 100 mM H_2O_2 for 10 min. The control and irradiated samples show sperm cells with halos, indicating a lack of DNA fragmentation. H_2O_2 induced sperm nucleus fragmentation, as evidenced by the absence of a halo. The graph represents an average of three different experiments ± SD at each time point. *Significance $P < 0.01$.

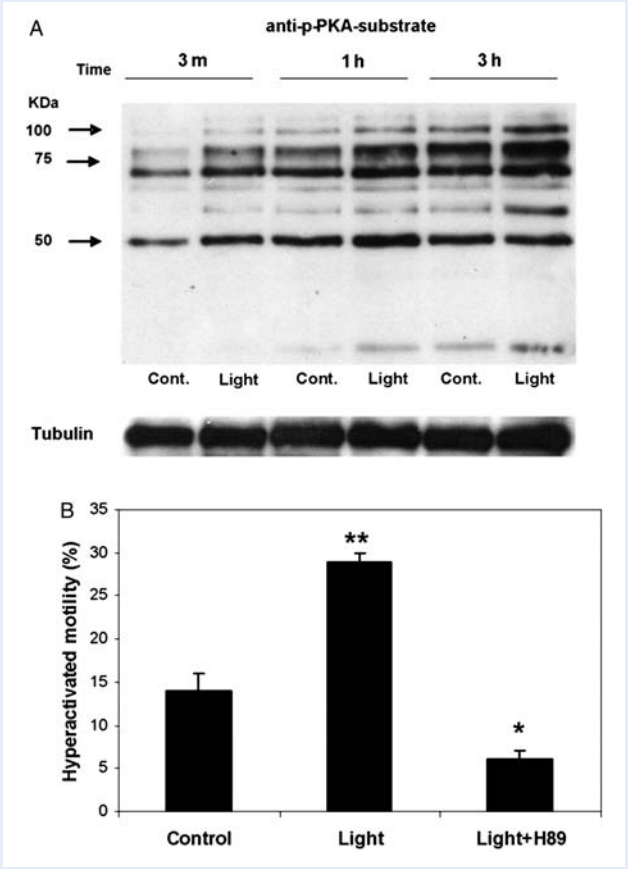

Figure 5 PKA activity and PKA-dependent HAM following light irradiation. (**A**) Western blot analysis of PKA activity during capacitation in irradiated and non-irradiated sperm. Cells were incubated in capacitation medium (F-10) for 3 h and irradiated for 3 min at the beginning of capacitation. Proteins were extracted at different times and subjected to SDS–PAGE followed by western blot analysis using anti-p-PKA-substrate and anti-Tubulin (loading control). (**B**) Effect of a PKA inhibitor on HAM: Cells were incubated with or without 50 μM H89 for 10 min and then irradiated for 3 min. HAM was measured 10 min after irradiation using CASA. The graph represents the average of six different experiments ± SE at each time. Asterisks indicate significance when compared with control *$P < 0.05$ **$P < 0.01$.

Light enhances calcium influx into the cells

In our previous study, we showed an increase in intracellular calcium in sperm irradiated with 640 nm (Cohen et al., 1998) or 780 nm (Lubart et al., 1992). Here, we showed that 2.5 min after sperm irradiation with visible light there is a significant increase in the fluorescence intensity indicated an increase in intracellular calcium concentration (Fig. 8A). Moreover, the light-mediated increase in HAM was significantly reduced when cells were incubated in calcium-deficient medium, containing the calcium chelator, EGTA (Fig. 8B), and in these conditions, very little increase in the fluorescence was observed

(not shown). Thus, the non-specific effect of light on Fluo-4 fluorescence is negligible and the main effect is due to the effect of light on calcium influx. The specificity of the light effect on calcium influx in sperm is further supported by our previous studies in which we used $^{45}Ca^{2+}$ to show the enhanced effect of light (Lubart *et al.*, 1992; Cohen *et al.*, 1998). It should also be mentioned that light irradiation enhanced HAM in the Fluo-4 loaded cells as in the control cells (not shown). The development of HAM in the presence of EGTA could be restored by elevating the free Ca^{2+} concentration (Fig. 8B), further supporting the importance of Ca^{2+} for the development of HAM. The induction of HAM by light was also significantly inhibited (50%) by the voltage-dependent-Ca^{2+}-channel (VDCC) blocker, nifedipine (Fig. 8B), suggesting that this calcium channel is activated by light irradiation. Similar inhibition (54%) by nifedipine was found in the control cells, suggesting that the VDCC mediates HAM in the cells. Interestingly, light irradiation caused 76% enhancement of HAM in the control cells and 100% enhancement in the nifedipine-treated sperm (Fig. 8B), suggesting that a Ca^{2+} channel other than VDCC might be involved in the effect of light on HAM.

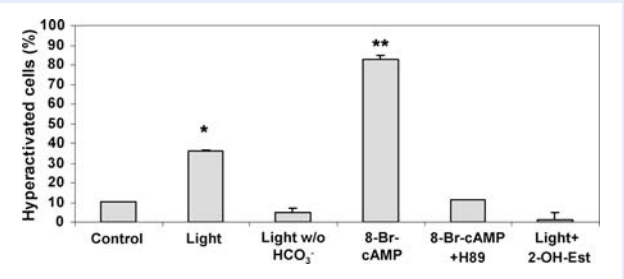

Figure 6 Involvement of cAMP in hyperactivation. Cells were washed and incubated in medium (F-10) with or without bicarbonate, 50 μM H89, or 20 μM 2-OH-estradiol, for 10 min. Then 1 mM 8-Br-cAMP was added to the relevant tubes for 3 min. Samples were irradiated for 3 min, and 10 min later HAM was measured using CASA. Graphs represent the average of five different experiments ± SE at each time point. Asterisks indicate significance when compared with control. $*P < 0.05$ $**P < 0.01$.

Discussion

We show here that 3 min light treatment of non-capacitated sperm caused a two-fold increase in their HAM compared with control cells; this high HAM continued for 3 h under capacitative conditions (Fig. 1B). The fact that this light treatment affects HAM (an indicator of sperm capacitation), and did not affect sperm total motility (Fig. 1A) suggests that light affects sperm capacitation. Further support for this conclusion is provided by our previous study showing that light irradiation of mouse sperm enhances the *in vitro* fertilization rate by 37% and increases protein tyrosine phosphorylation (Cohen *et al.*, 1998), a process that occurs as part of sperm capacitation (Visconti *et al.*, 1999). The effect of light on sperm capacitation is supported further in this study by showing that light irradiation enhances ROS production and HAM, two capacitation parameters. It is known that visible light can be absorbed by cellular photosensitizers such as cytochromes, flavins/riboflavins (Laloraya *et al.*, 1994) and NADPH (Cunningham *et al.*, 1985). Absorption of light by these photosensitizers causes their excitation, followed by electron transfer to O_2, thereby generating ROS. There is some evidence that ROS are formed as part of animal sperm capacitation, and ROS scavengers inhibit the capacitation process (Leclerc *et al.*, 1997). Moreover, there is evidence for mitochondrial ROS generation in human spermatozoa (Koppers *et al.*, 2008). We showed here that light illumination enhanced ROS formation in the sperm, especially in the midpiece where the mitochondria are located (Fig. 3A and B). In addition, treatment of sperm with SOD or azide prevented the increase in HAM in the light treated or control cells (Fig. 2), indicating that the super-oxide anion is formed during the incubation time, and mediates sperm HAM. Moreover, we showed (Fig. 3) that blocking mitochondrial respiration by azide causes a significant reduction in ROS production, indicating that most of the ROS produced by light illumination is of mitochondrial origin. Since in our washed sperm we could not detect any leukocytes, we assume that most of the

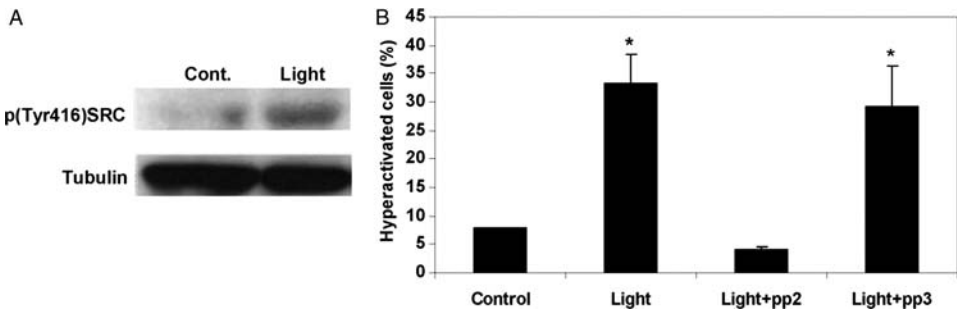

Figure 7 Src activity following light irradiation. (**A**) Western blot showing Src activity during capacitation in irradiated and non-irradiated sperm. Cells were incubated in capacitation medium (F-10) and irradiated for 3 min at the beginning of capacitation. Proteins were extracted 10 min after irradiation and subjected to SDS–PAGE followed by western blot analysis using anti-p-Src and anti-Tubulin. (**B**) Cells were incubated with or without 10 μM pp2 or pp3 for 10 min, and then irradiated for 3 min. HAM was measured 10 min after irradiation using CASA. Graph represents the average of three independent experiments ± SE at each time. $*P < 0.01$.

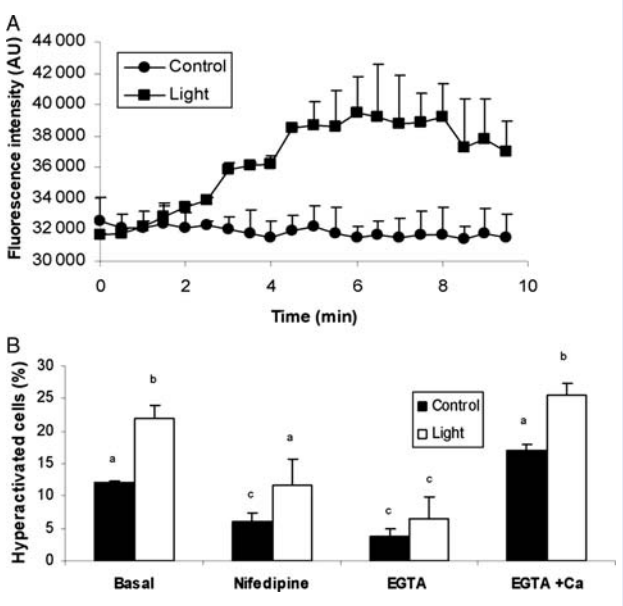

Figure 8 Measurement of Ca^{2+} influx and Ca^{2+} influx-dependent HAM in light-irradiated sperm cells. (**A**) Sperm cells were incubated with Fluo-4/AM as described and then irradiated for 3 min. Fluorescence was measured immediately after irradiation, indicating calcium influx, for a period of 10 min. (**B**) Cells were incubated with 1 mM EGTA or 30 μM nifedipine with or without Ca^{2+}. After 10 min, cells were irradiated for 3 min and HAM was measured 10 min after irradiation using CASA. The graphs shows the average ± SE of three different experiments from different donors. Different letters indicates significance using Duncan test ($P < 0.05$).

ROS produced by light illumination is generated by sperm mitochondria, as shown in Fig. 3B.

Another important process that occurs as part of mammalian sperm capacitation is PKA-dependent protein tyrosine phosphorylation (Visconti *et al.*, 1999). Light treatment enhanced PKA activity (Fig. 5A) and the PKA inhibitor H89 (Rotman *et al.*, 2010) inhibited this effect, as well as light-induced HAM (Fig. 5B). Moreover, the enhanced effect of light on HAM could not be seen when sAC activation was prevented by incubating the sperm in bicarbonate-deficient medium or with 2-OH-Estradiol (Fig. 6); the effect of light could be recovered by adding 8-Br-cAMP (Fig. 6). Moreover, the enhanced effect of 8Br-cAMP on HAM was not inhibited by SOD (Fig. 2), indicating that ROS mediates cAMP production. Together, these data clearly indicate that light enhances sAC/PKA-dependent HAM in human sperm. Moreover, the development of HAM in human sperm capacitation depends on sAC/cAMP/PKA activities. Although Suarez and Marques showed in bovine sperm that procaine- or caffeine-induced hyperactivation is independent of PKA activity (Marquez and Suarez, 2004), other studies showed that PKA is necessary for hyperactivation development in several other species (Nolan *et al.*, 2004; Baker *et al.*, 2006; Harayama and Nakamura, 2008; Kaneto *et al.*, 2008). Apparently the involvement of PKA in the development of HAM depends on the species. It is well established that sperm capacitation is mediated by PKA activity, and HAM is developed under capacitation conditions. Thus, it is likely that PKA activity is indeed involved in the development of HAM.

We and others have recently suggested that PKA can activate sperm Src-family kinase, which can further mediate the sperm capacitation processes (Baker *et al.*, 2006; Etkovitz *et al.*, 2009). Here we showed that light treatment enhances Src phosphorylation/activation, and that the Src inhibitor PP2 blocks HAM induced by light and reduces the baseline HAM in control cells (Fig. 7).

Another important question relates to the role of intracellular calcium in regulating sperm HAM. We showed previously that light irradiation enhances the intracellular calcium concentration in mouse sperm (Cohen *et al.*, 1998) as well as calcium influx in bovine sperm (Lubart *et al.*, 1992). Moreover, it has been demonstrated that extracellular Ca^{2+} is required to preserve hyperactivation *in vitro* (Yanagimachi, 1982; Fraser, 1987) and that the concentration of intracellular free calcium ions is higher in hyper-activated sperm than in non-hyper-activated sperm (Suarez *et al.*, 1993).

We showed here that the Ca^{2+} influx is elevated very rapidly in irradiated cells (Fig. 8A). Additionally, HAM did not develop when cells were incubated in Ca^{2+}-deficient medium (Fig. 8B). It is known that sAC is activated by Ca^{2+}; thus these data further supporting our notion regarding the involvement of sAC/PKA in the mechanism by which light enhances HAM. Moreover, the effect of light on HAM could be blocked by the VDCC blocker, nifedipine (Fig. 8B). These data clearly indicate that the development of HAM depends on calcium influx into the cells, and that light treatment enhances HAM probably by activating VDCC.

In summary, the presented data provide an interesting mechanism for light effects on biological systems, in general, and on sperm cells, in particular. Moreover, the results have great importance because visible light irradiation is an available simple process that can be used in case of a deficiency in sperm to undergo complete capacitation. Light irradiation can be offered as a preliminary treatment for sperm cells, which will be used for IVF (*in vitro* fertilization) or intrauterine insemination.

Authors' roles

S.S. conducted the experiments, analyzed data and wrote the paper; A.W. and A.S. (Head of IVF) supplied sperm; D.I. helped in conducting experiments. R.L. supplied the lamp and H.B. (Head of group) supplied the budget, analyzed data and wrote the paper.

Funding

Ihel Foundation grant to H.B.

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