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Involvement of A_1 Adenosine Receptors in the Acquisition of Fertilizing Capacity

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ABSTRACT: Ejaculated mammalian spermatozoa acquire competence to fertilize oocytes by a two-step process: capacitation followed by acrosome reaction. The biochemical and biophysical modifications occurring in vivo in the female reproductive tract can be reproduced in vitro, and previous studies have suggested a capacitative role for adenosine A_1 receptor (A_1R). Mice with a targeted disruption of the Adora 1 gene ($A_1R-/-$ mice) provide a useful model for better understanding the role of the A_1R in fertility. Murine spermatozoa express A_1R in the head, neck, midpiece region, and tail. The number of capacitated spermatozoa incubated in human tubal fluid was significantly reduced in $A_1R-/-$ compared with $A_1R+/+$ and $A_1R+/-$ spermatozoa. The difference between A_1 $R+/+$ and $A_1R-/-$ mouse spermatozoa was mainly in the time necessary to reach the maximum percentage of capacitation. $A_1R+/+$ murine sperm obtained the full state of capacitation within 90 minutes whereas $A_1R-/-$ sperm required 240 minutes. Caffeine, a known antagonist of A_1 and A_{2A} adenosine receptors, lowered the number of capacitated sperm and affected the time of capacitation in a dose-dependent manner, mimicking the effects of the lack of A_1 receptors. Although number, motility, and viability of $A_1R-/-$ murine sperm was not significantly different from $A_1R+/+$ mouse spermatozoa, a significant reduction of the number of pups produced by $A_1R-/-$ male mice suggests that A_1 receptors must be fully operative to accomplish the optimal degree of capacitation and thereby fertilization.

Key words: A_1 adenosine receptors KO mice, A_1 adenosine receptors mouse sperm localization, capacitation, caffeine, fertility.

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Mammalian spermatozoa emerging from the male reproductive tract are incapable of fertilizing eggs. They acquire this ability either during transit in the female reproductive tract (Yanagimachi, 1994) or during incubation in suitable in vitro media (Allegrucci et al, 2001). Such conditioning, called capacitation, renders the spermatozoa capable of interaction with the oocyte and thereby induces the acrosome reaction (AR). Capacitation and AR are related to many effectors and signal transduction pathways, but the molecular basis of these processes is still only partially known (Florman and Babcock, 1991; Kopf et al, 1991; de Lamirande et al, 1997; Tulsiani et al, 1998; Thundathil et al, 2002). Sperm capacitation is a multistep process that involves several biochemical and ultrastructural changes in the sperm membrane, ranging from modification of membrane lipid composition to an increased permeability to ions. The efflux of membrane cholesterol leads to bovine sperm capacitation (Cross, 1998). Albumin, high-density lipoproteins (HDLs), and follicular and oviductal lipoproteins are capacitation effectors of human and bovine spermatozoa (Therien et al, 1998; Therien et al, 2001). It has been shown that capacitation is correlated with an increase of protein tyrosine phosphorylation (Visconti et al, 1995a; Visconti et al, 1995b; Visconti et al, 1999; Aitken et al, 1998). The signal transduction pathway leading to protein tyrosine phosphorylation is thought to be central to the attainment of the capacitative state (Visconti and Kopf, 1998) and the concomitant expression of hyperactivated motility (Mahoney and Gwathmey, 1999; Si and Okuno, 1999).

Capacitation and increased motility are related to an increase of protein tyrosine phosphorylation (Visconti et al, 1995a; Visconti et al, 1995b; Mahoney and Gwathmey, 1999; Si and Okuno, 1999). In addition, several proteins undergo serine/threonine phosphorylation or threonine/tyrosine double phosphorylation as capacitation proceeds (Naz, 1999; Thundathil, 2002).

Adenosine is capable of modulating the activation of mammalian spermatozoa by interaction with specific transmembrane receptors (Minelli et al, 1995; Fenichel et al, 1996; Minelli et al, 2000; Allegrucci et al, 2001).

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N^6-cyclopentyladenosine (CPA), a highly specific adenosine A_1 receptor agonist, has a capacitative effect on human spermatozoa with a concomitant increase of tyrosine phosphorylation in a subset of proteins that are human homologues of mouse capacitated state indicators (Carrera et al, 1996; Brewis et al, 1998; Osheroff et al, 1999; Allegrucci et al, 2001). The purpose of the present study is to further investigate the role of A_1 receptors in the acquisition of the fertilizing capacity. Mice with a targeted disruption of the Adora 1 gene provide a useful model for these studies, and using spermatozoa from such mice, we have examined capacitation in vitro and fertility in vivo.

Materials and Methods

Adora 1 (A_1R) Knockout Mice

The Adora 1 gene coding for the adenosine A_1 receptor was inactivated as described elsewhere (Johansson et al, 2001). Animals, backcrossed twice against C57Bl6 and aged between 4 and 14 weeks, were used in the present study, and all experiments were conducted using protocols following EUR directives, approved by the Institutional Animal Care and Use Committee of Perugia University.

Sperm Preparation and Capacitation

Epididymal spermatozoa were extracted from adult mice aged between 4 and 14 weeks. Mice were killed by cervical dislocation. The epididymis was removed from the animals, caput regions were separated from cauda regions and the latter were carefully blotted on moist filter paper, separated from blood clots and extraneous tissue before suspension in 10 mL of human tubal fluid (HTF, Irvine Scientific, Santa Ana, Calif) that had been prewarmed to 37°C. To reduce epididymal debris contamination, the epididymes were gently squeezed and punctured rather than minced. The spermatozoa were immediately placed in an incubator at 37°C and left for a short time to allow the spermatozoa to disperse throughout the media. The number of spermatozoa was determined by Thoma chamber (Brand Gmbh Co, Werteim/Main, Germany) and adjusted to a concentration of $10x10^6$/mL HTF. Sperm were then incubated in HTF for 90 minutes at 37°C in an incubator with a moist 5% CO_2/95% air atmosphere to permit capacitation. Viability was evaluated by fluorescent microscopy with an Axioplan Zeiss microscope (Gottingen, Germany) with 5-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI). Sperm motility was determined by examination using phase contrast microscopy (at least 100 sperm/sample at 400× magnification). A subjective score was recorded for sperm motility (Thomas and Meizel, 1988) using a scale of 1 (twitching, no progressive motion) to 4 (vigorous forward motility). Usually, $12 \pm 2 \times 10^6$ sperm/mL with 73 ± 8% motility (scale 4) and 78 ± 7% viability were obtained from one mouse.

Preparation and Solubilization of Mouse Zonae Pellucidae

ZP were prepared from homogenized ovaries of 22-day-old virgin female as described by Ward et al (1992). The zonae pellucidae (ZP), which were stored at −80°C in aliquots in a buffer containing 25 mM triethanolamine, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Polyvinylpyrrolidone, pH 7.8, were centrifuged and then resuspended in a buffer containing 20 mM Tris-HCl, 130 mM NaCl, pH 7.4, at final concentration of 200 μg/mL of ZP. Based on a protein content of 4.8 ng/mouse ZP, 200 μg/mL of ZP is the equivalent of 40 ZP/μL (Bleil and Wasserman, 1980). The purity of the ZP preparations was determined by SDS-PAGE followed by Silver Staining. ZP were then solubilized at 60°C for 1 hour, centrifuged at 15,000 × *g* for 2 minutes at 24°C to remove particulate insoluble material, and the supernatant incubated immediately with capacitated sperm for 30 minutes at final concentration of 2 ZP/μL of sperm suspension.

Evaluation of Acrosomal Status

The ability of the spermatozoa to respond to ZP and undergo an acrosome reaction was utilized as an assay for capacitation (Florman and Babcock, 1991; Visconti et al, 1998; Osheroff et al, 1999). It has been demonstrated that the ZP-induced acrosome reaction occurs only in capacitated spermatozoa (Shi and Roldan, 1995). 10×10^6 sperm, incubated for 90 minutes in HTF at 37°C in 5% CO_2/95% humidified air, were assessed for the capacitated state following incubation of the sperm with 2 ZP/μL for 30 minutes. In evaluating the effects of caffeine on the acquisition of the capacitated status, caffeine was added to HTF and incubated under the described experimental conditions. Prior to drying and staining, randomly selected slides containing ~ 10^5 cells were examined to verify sperm motility and viability. The acrosomal status of the sperm was determined on air-dried sperm smears by Coomassie blue staining (Miller et al, 1993; Sato et al, 2000). At least 200 cells were scored with an Axioplan Zeiss microscope (Gottingen, Germany). The percentage of capacitation was assessed by subtracting spontaneous acrosome reaction % (SAR) to ZP-induced acrosome reaction % (ZP-IAR) at each indicated time.

Indirect Immunofluorescence

Samples for confocal microscopy analyses were prepared as follows: sperm were fixed in 3.7% paraformaldehyde, washed in PBS, and permeabilized for 10 minutes in 0.2% Triton X-100 at room temperature. To block nonspecific sites, samples were incubated for 1 hour in 10% horse serum in PBS-1% BSA. Sperm were then incubated with PC21 (70 μg/mL) overnight at 4°C, washed, and incubated in PBS-BSA with Alexa Fluor 488 antirabbit IgG antibodies (1:100) for 40 minutes. Affinity-purified polyclonal antibody against A_1R (PC21) was a gift of Prof Franco (Barcelona, Spain) and characterized elsewhere (Ciruela et al, 1995). Alexa Fluor 488 antirabbit IgG were purchased from Molecular Probes (Eugene, Ore). Negative controls were prepared by omitting the first antibody. Confocal analysis was performed on an Olympus IMT2 microscope equipped with a Bio-Rad MRC 1024 (Bio-Rad Laboratories, Hercules, Calif).

Samples for fluorescence microscopy were prepared as described, using a goat polyclonal adenosine A_1 receptor (C-19) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) as primary antibody. Antigoat IgG-tetramethylrhodamine isothiocyanate (TRITC) was purchased by Sigma (St Louis, Mo). Negative con-

*Effects of male genotype**

Parameter	A_1R +/+	A_1R +/−	A_1R −/−
A. In vivo fertility			
Number of litters	40	40	40
Average number of pups	8 ± 2.1	8 ± 1.9	5 ± 1.7
Birthweight (g)	2.07 ± 0.8	2.05 ± 0.6	2.08 ± 0.4
Days between litters	45 ± 8	47 ± 8	53 ± 15
B. Reproductive parameters			
Number of spermatozoa	$13 \times 10^6 \pm 2 \times 10^6$	$11 \times 10^6 \pm 3 \times 10^6$	$12 \times 10^6 \pm 1 \times 10^6$
% Viability	80 ± 10	76 ± 7	77 ± 9
% Motility	75 ± 7	72 ± 9	71 ± 11
C. Phenotype			
Weight of adult animal (g)	33 ± 5	31 ± 7	32 ± 4
Weight of testis (mg)	132 ± 21	121 ± 15	122 ± 18

* A values are the means ± SEM of 40 litters, $P < .05$. B and C values are the means ± SEM of 20 male mice, $P < .05$.

trols were prepared using the peptide (sc-7500) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif)-blocked antibody. Samples were analyzed with a BX60 (Olympus Optical Co, Hamburg, Germany) epifluorescence microscope equipped with a standard rhodamine filter using a 100× objective.

In Vivo Studies

A_1R+/+, A_1R+/− and A_1R−/− males were mated with A_1R+/+ females. Three groups, each consisting of four males and eight females, were followed to forty pregnancies. Interlitter intervals, mean number and weight of pups, reproductive parameters, and phenotype characteristics were statistically analyzed.

Statistical Analysis

Data were expressed as means ± SEM. Statistical significance was determined by using Student's *t* test and ANOVA. A *P* value < .05 was considered significant.

Results

In Vivo Analysis

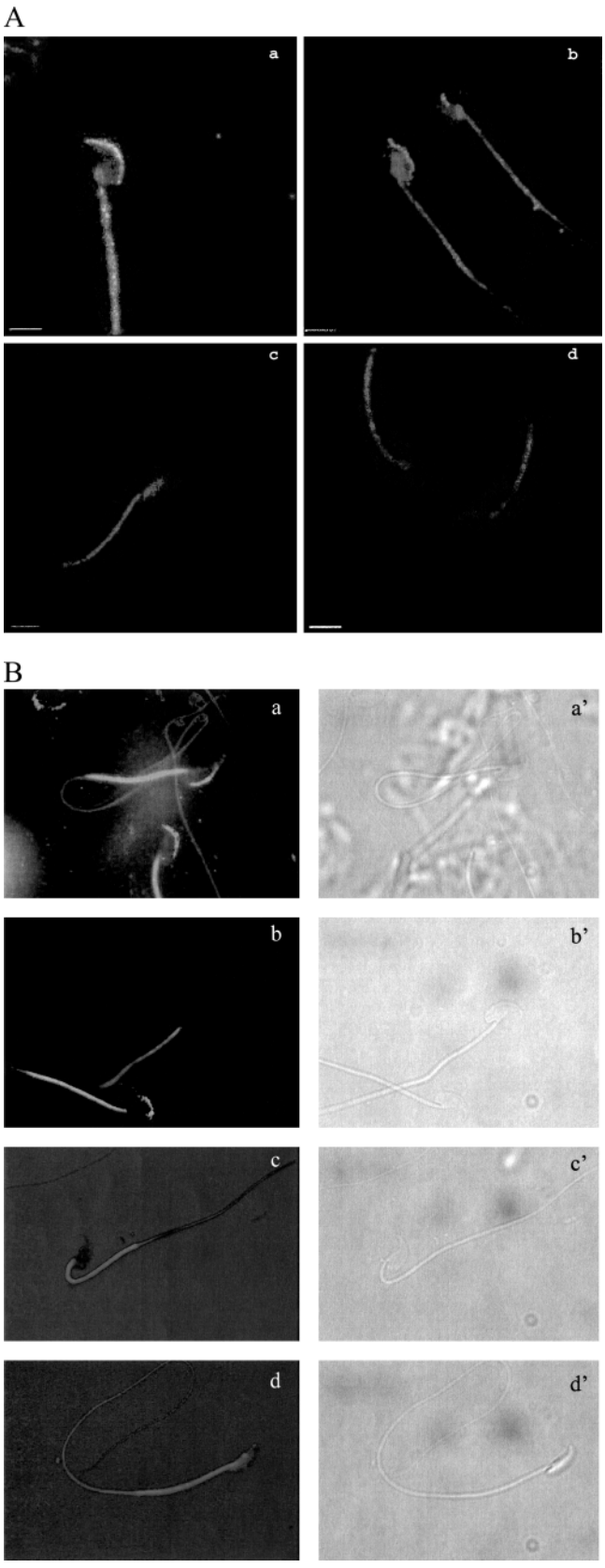
The study was carried out with three groups of animals, each characterized by male genotype, that is, males of the A_1R+/+, A_1R+/−, and A_1R−/− genotypes were mated with females of A_1R+/+ genotype. The average number, weight of pups, and interlitter intervals were recorded for 40 litters, reproductive parameters and phenotype characteristics were analyzed in males of each group (Table). The average number of pups was significantly reduced when A1R−/− male mice were used, whereas the average weight of pups was not modified. The interval between litters tended to be higher in A_1R−/− male mice than when male mice of other genotypes were used, but this result was not statistically significant. Reproductive parameters and phenotype showed no significant variations among genotypes, although small changes in motility might affect in vivo fertilization.

Localization of A_1R in Mice Spermatozoa

The localization of A_1R was examined using a specific anti-A_1 antibody in A_1R+/+, A_1R+/−, and A_1R−/− mouse spermatozoa by confocal microscopy (Figure 1A). A_1 receptor was preferentially localized in the acrosome region, in the neck, the midpiece, and the tail of A_1R+/+ (panel a) and A_1R+/− (panel b) mouse spermatozoa. In A_1R−/− spermatozoa (panel c) only a weak and nonspecific fluorescence was visible, which could be ascribed to the secondary antibody (panel d). PC21 antibody appears to be highly selective toward A_1R since its binding was eliminated in A_1R−/− mice spermatozoa. Fluorescence microscopy images (Figure 1B) were in agreement with data of confocal microscopy. Indeed, A_1R+/+ (panel a) and A_1R+/− (panel b) mouse spermatozoa showed a strong fluorescence in the acrosome region, in the neck and midpiece, whereas only a weak signal was localized in the tail. In A_1R−/− spermatozoa (panel c) only a weak and nonspecific fluorescence was visible in the midpiece region. The same signal at the midpiece region was observed in control samples (panel d).

Capacitative Status of Mice Spermatozoa

Sperm samples from A_1R+/+, A_1R+/−, and A_1R−/− mice, incubated in HTF for 90 minutes at 37°C in 5% CO_2, were assessed for the capacitated state by the ability to undergo the ZP-induced acrosome reaction following incubation of the sperm with 2 ZP/μL for 30 minutes. Comassie blue staining was used to assess the acrosomal status. Results are shown in Figure 2. A_1R+/+ and A_1R+/− mouse spermatozoa did not show significant differences in the percentage of ZP-induced acrosome reacted cells, whereas a significant difference could be observed between the A_1R+/+ and A_1R−/− mice (Figure 2A). The spontaneous AR percentage of sperm preparations after 90 minutes incubation at 37°C in 5% CO_2 in HTF was the same



in each group, meaning that the incidence of spontaneous AR did not vary among genotypes (Figure 2B).

The time necessary to attain the maximum percentage of capacitated cells was different in $A_1R+/+$ and $A_1R-/-$ mouse spermatozoa (Figure 2C).

$A_1R+/+$ murine sperm attained the full state of capacitation within 90 minutes, whereas $A_1R-/-$ sperm showed a delay, since 240 minutes was necessary to attain the maximum percentage of ZP-acrosome reacted cells. At 300 minutes' incubation, both samples showed a decreased number of capacitated cells, mainly due to the increased number of spontaneous AR (data not shown). The ability to undergo ZP-induced acrosome reaction was examined in the presence of caffeine, a known antagonist of A_1 and A_{2a} adenosine receptors (Fredholm et al, 1999). Sperm samples were prepared from $A_1R+/+$, $A_1R+/-$ and $A_1R-/-$ mice as previously described and incubated in HTF and caffeine, at 15 and 100 μM, for 90 minutes at 37°C in 5%CO_2. Results are shown in Figure 3. $A_1R+/+$ sperm capacitated in the presence of 15 μM caffeine showed a ZP-induced, acrosome-reacted cell percentage that was not significantly different from $A_1R+/-$ murine sperm. When the capacitation of $A_1R+/+$ mouse sperm was carried out in the presence of 100 μM caffeine, the percentage of ZP-acrosome reacted cells was significantly different from the control and close to that observed in $A_1R-/-$ mouse sperm. Heterozygous mouse sperm $A_1R+/-$ responded to 15 μM caffeine with a ZP-acrosome reacted percentage significantly different from the control and similar to that observed in $A_1R-/-$ mouse sperm. The effect of 100 μM caffeine was almost the same as that of 15 μM caffeine. The percentage of ZP-acrosome reacted cells was not affected by caffeine in experiments with $A_1R-/-$ sperm (Figure 3A). Caffeine, at the used concentrations, did not induce increases of

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Figure 1. Localization of A_1R in murine spermatozoa. **(A)** Spermatozoa were adhered to glass coverslips, fixed and permeabilized. The immunofluorescence staining was performed as described in ''Materials and Methods,'' using PC21 (70 μg/mL) as primary antibody and Alexa Fluor 488 anti-rabbit IgG (Molecular Probes, Eugene, Ore). Fluorescence at the cell surface was observed by confocal microscopy. The experiments were repeated (n = 7) using sperm from different mice, representative images corresponding to a horizontal section of the cells are shown. Panel a: $A_1R+/+$ mouse sperm; panel b: $A_1R+/-$ mouse sperm; panel c: $A_1R-/-$ mouse sperm; panel d: cells incubated with secondary antibody only. Bar = 5 μm. **(B)** Fluorescence microscopy images. The immunofluorescence staining was performed as described in ''Materials and Methods,'' using goat polyclonal adenosine A_1 (Santa Cruz Biotechnology, Santa Cruz, Calif) as primary antibody and antigoat IgG- TRITC (Sigma, St Louis, Mo). Observations were done using fluorescence and phase-contrast microscope with a 100× objective. The experiments were repeated (n = 3) using spermatozoa from different mice and a representative experiments is shown. Panels a, b, c, and d: fluorescence microscopy images of $A_1R+/+$ mouse sperm, $A_1R+/-$ mouse sperm, $A_1R-/-$ mouse sperm, and control; Panel a′, b′, c′, and d′: corresponding phase-contrast images.

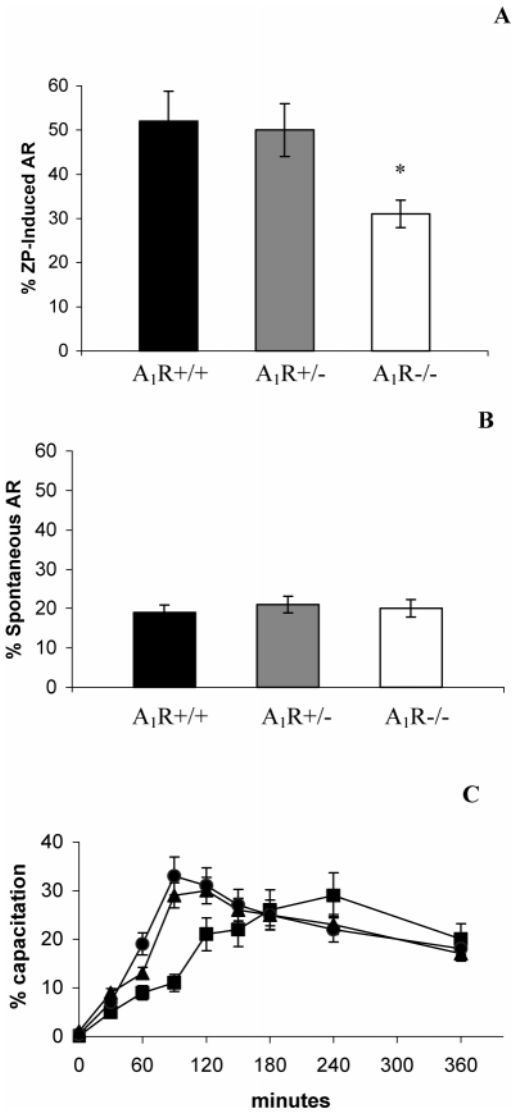

Figure 2. Capacitation of $A_1R+/+$, $A_1R+/-$, and $A_1R-/-$ mouse spermatozoa. Spermatozoa were incubated in HTF for 90 minutes at 37°C in 5% CO_2. AR was induced by ZP (2 ZP/μL for 30 minutes at 37°C). Acrosome-reacted cells were determined with Coomassie blue staining. Results represent the mean ± SEM of n = 7 independent experiments performed in triplicate and 200 sperm counted/sample. The asterisk (*) indicates a significant difference vs. Control, $P < .05$. **(2A)** ZP-induced AR percentage of the samples after 90 minutes incubation under the described experimental conditions. **(2B)** Spontaneous AR percentage of the samples after 90 minutes incubation under the described experimental conditions. **(2C)** Percentage of capacitated cells as function of incubation time in $A_1R+/+$ (●), $A_1R+/-$ (▲), and $A_1R-/-$ (■) mouse spermatozoa. Sperm were incubated under the described experimental conditions and aliquots withdrawn at the indicated times.

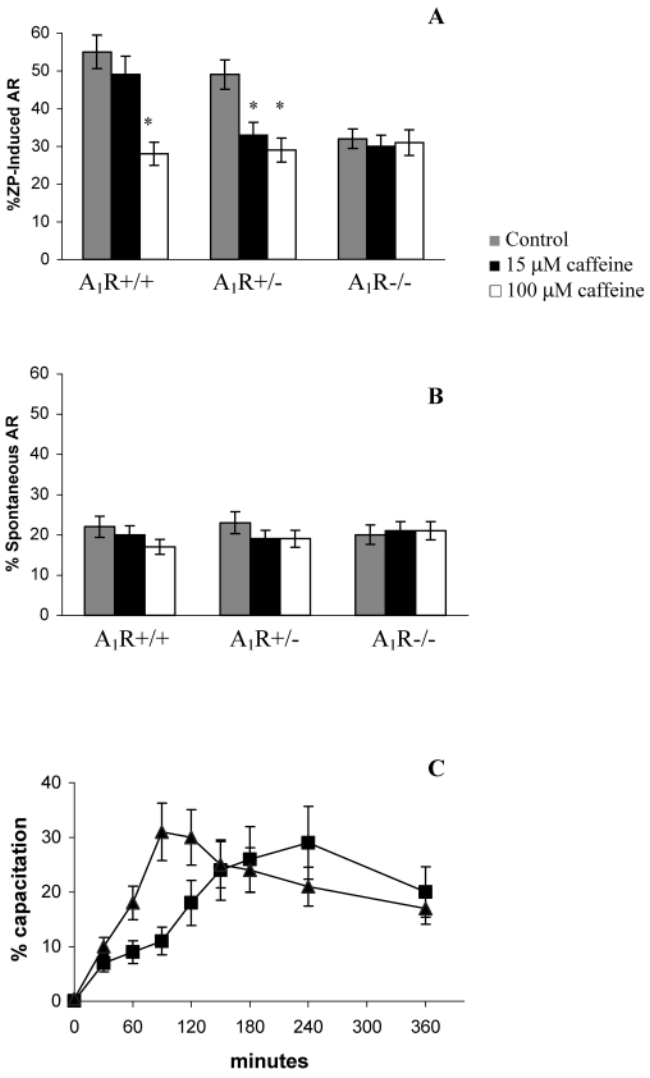

Figure 3. Effects of caffeine on capacitation of $A_1R+/+$, $A_1R+/-$, and $A_1R-/-$ mouse spermatozoa. Spermatozoa were incubated in HTF in the presence of 15 μM and 100 μM caffeine for 90 minutes at 37°C in 5% CO_2. AR was induced by ZP (2 ZP/μL for 30 minutes at 37°C). Acrosome-reacted cells were determined with Coomassie blue staining. Results represent the mean +/− SEM of n = 7 independent experiments performed in triplicate and 200 sperm counted/sample. The asterisk (*) indicates a significant difference vs Control ($P < .05$). **(3A)** Percentage of ZP-acrosome reacted spermatozoa after 90 minutes incubation under the described experimental conditions; **(3B)** spontaneous AR percentage of the samples after the 90 minutes incubation. **(3C)** Percentage of capacitated cells as function of incubation time of $A_1R+/+$ murine sperm in the presence of 15 μM caffeine (▲) and 100 μM caffeine (■). Sperm were incubated under the described experimental conditions and aliquots withdrawn at the indicated times.

spontaneous AR percentage in $A_1R+/+$, $A_1R+/-$, and $A_1R-/-$ mouse spermatozoa (Figure 3B). The effects of caffeine on the time necessary to attain the maximum percentage of capacitation was investigated in $A_1R+/+$ mouse spermatozoa. Results in the presence of 15 μM caffeine showed that this concentration of antagonist reproduces the situation found in $A_1R+/-$ murine spermatozoa. In the presence of 100 μM caffeine, the time necessary to attain the maximum percentage of capacitated cells was increased as in $A_1R-/-$ mouse spermatozoa (Figure 3C). The same results were obtained in the presence of caffeine when spermatozoa were washed prior to adding ZP (data not shown), meaning that caffeine did not affect ZP-IAR.

Discussion

In this study we have provided evidence that sperm lacking the A_1 receptors are less responsive to capacitating effectors and that there is a significant reduction in the number of pups produced by $A_1R-/-$ male mice, although reproductive parameters, that is, number, motility, and viability, of caudal epididymal spermatozoa from $A_1R-/-$ mice do not show significant differences compared with $A_1R+/+$ mice. Although there are data in the literature reporting the involvment of A_{2A} receptors in sperm motility, cAMP production, and protein phosphorylation (Fraser and Duncan, 1993; Shen et al, 1993; Fenichel et al, 1996), we have previously shown that the stimulation of A_1 receptor has a capacitative effect on human spermatozoa (Minelli et al, 2000; Allegrucci et al, 2001). Therefore, signaling initiated by A_1 receptors in response to either synthetic or endogenous agonists plays a role in promoting the attainment of capacitative status of spermatozoa. The capacitation percentage, that is, the difference between ZP-induced AR and spontaneous AR, of $A_1R+/-$ murine sperm is only slightly and not significantly reduced compared with that of $A_1R+/+$ sperm, whereas significant differences in the percentage of capacitation are found in $A_1R-/-$ sperm. The attainment of the maximum percentage of capacitation is also greatly delayed in mice spermatozoa lacking the A_1 receptors. $A_1R+/+$ murine spermatozoa, treated with a high dose of caffeine, need 3 times longer to reach the maximum percentage of capacitation than untreated $A_1R+/+$, a similar length of time to that observed in $A_1R-/-$ mouse sperm. Therefore, the total blocking of A_1 receptors reproduces the situation found in knockout mice. This could be relevant for understanding the possible effects of caffeine on fertility. It has been found that 15 μM caffeine, an amount seen in body fluids after human consumption of 3 to 6 cups of coffee per day, is likely to bind to and inhibit half of the adenosine receptors (Fredholm et al, 1999). The present data, showing that this dose of caffeine had the same very low effect as that observed in $A_1R+/-$ mice, is entirely compatible with the previous results. By contrast, a very high dose of caffeine, 100 μM, did cause a significant reduction in sperm capacitation. However, concentrations of caffeine of this magnitude are unlikely to be reached by caffeine consumers. Indeed, strong side effects would preclude ingestion of this amount. Hence, our data suggest that regular caffeine consumption is unlikely to significantly affect spermatozoa function. This is reassuring for coffee drinkers. Interestingly, the lower concentration of caffeine had a clearly inhibitory effect on spermatozoa from $A_1R+/-$ mice, which have a reduced number of A_1 receptors. It may be that individuals with low adenosine receptor number could be more susceptible to the inhibitory effects of caffeine on male fertility.

In conclusion, we have shown that the lack of expression of A_1 receptors is associated with delayed capacitation. Very high doses of caffeine, unlikely to be reached by coffee drinkers due to strong side effects, mimic the lack of expression of A_1 receptors, whereas caffeine, at levels achieved by regular human consumption, appears to minimally affect sperm capacitation. Our in vivo results show that $A_1R-/-$ mice are less fertile, implying that A_1 receptors, although not indispensable for the completion of capacitation, are involved in the efficiency of the process. The reduced number of offspring indicates that several signaling pathways, including those activated by adenosine A_1 receptors, must be interactive and fully operative to accomplish the maximum degree of capacitation and hence fertility.

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