Journal of Andrology, Vol. 24, No. 3, May/June 2003

Effect of Azorellanone, a Diterpene From *Azorella yareta* Hauman, on Human Sperm Physiology

PATRICIO MORALES,* MILENE KONG,* EDUARDO PIZARRO,* CONSUELO PASTEN,* GLAUCO MORALES,† JORGE BORQUEZ,† AND LUIS A. LOYOLA†

*From the *Unit of Reproductive Biology, Faculty of Health Sciences, and the †Chemistry Department, Faculty of Basic Sciences, University of Antofagasta, Antofagasta, Chile.*

ABSTRACT: Previous studies have shown that cyclic terpenes extracted from plants decrease sperm motility and concentration in rats. In this work, we studied the effect 13-α-hydroxy-7-oxoazorellano (azorellanone), a cyclic diterpene extracted from *Azorella yareta* Hauman, on in vitro human sperm physiology. Sperm aliquots, capacitated for 4.5 or 20 hours, were incubated for 15 minutes with different concentrations of azorellanone. Then, the effects of azorellanone on sperm motility, viability, binding to the human zona pellucida, progesterone-induced acrosome reactions and increase in intracellular Ca^{2+} concentration, and trypsin- and chymotrypsin-like protease activities were evaluated. Sperm motility was evaluated according to World Health Organization (WHO) guidelines; sperm viability with the supravital dye Hoescht 33258; sperm–zona binding with the hemizona assay; progesterone-induced acrosome reaction with fluorescent lectin; intracellular Ca^{2+} level with fura 2; and protease activity with the synthetic substrates N-t-Boc-Gln-Ala-Arg-Amido-4-methylcoumaryn and Succinyl-Leu-Leu-Val-Tyr-Amido-4-methylcoumaryn. The results obtained indicate that azorellanone inhibited sperm motility in a concentration-dependent manner at 0.15, 1.5, and 3 mM, while sperm viability was only inhibited at 3 mM. Treatment with azorellanone significantly inhibited sperm–zona binding, progesterone-induced acrosome reactions, and intracellular Ca^{2+} concentration. Treatment of free-swimming sperm with azorellanone did not affect protease activity; however, the incubation of sperm extracts with azorellanone significantly inhibited both trypsin-like and chymotrypsin-like protease activities. In conclusion, azorellanone has a significant effect on the different parameters that characterize human sperm physiology.

Key words: Sperm–zona binding, cyclic terpenes, intracellular Ca^{2+}, protease activity.

J Androl 2003;24:364–370

There is a great deal of interest in searching for a contraceptive method to inhibit, in a reversible and specific manner, the functions of the male gamete. The investigations about compounds of vegetal origin have aroused an increasing interest in the pharmaceutical industry, mostly because of the success in the treatment of several diseases and their possible applications as contraceptive agents. Thus, a variety of compounds of vegetal origin have been reported to affect sperm function, both during spermatogenesis and at a posttesticular level. Examples of these compounds are gossypol, a polyphenol compound, extracted from the cotton plant (National Coordinating Group on Male Antifertility Agents, 1978), extracts from *Montanoa frutescens* (Ponce-Monter et al, 1985), and triptolide, a diterpene epoxide, extracted from the Chinese medicinal plant *Tripterigium wilfordii* (Lue et al, 1998; Sinha Hikim et al, 2000).

In the high Andes in the north of Chile grows *Azorella yareta* Hauman, a shrub species that belongs to the Umbeliferae family. It is known as ''yareta,'' and it has been used as an herb medicine. Historical records indicate that yareta has been used to treat the common cold and ache, to reduce sugar in the blood, and possibly as an ointment to treat dermatological disorders. This species constitutes a valuable source of mulinane and azorellane diterpenoids (Loyola et al, 1997, 1998a,b). In a previous communication, we reported the structures of the mulinane derivatives mulinolic acid and mulin-11,13-dien-20-oic acid and those of the azorellane derivatives azorellanol, 13-α-hydrozyazorellane, and 13-β-hydrozyazorellane, which were obtained from a petroleum ether (petrol) extract of *A yareta* Hauman (Loyola et al, 2001).

In this report, we studied the more polar chromatographic fractions of the same extract and described the isolation of a new diterpenoid with an azorellane skeleton, the structure of which was established by spectroscopic analysis and by oxidation of 7-desacetylazorellanol 1, to our compound 13-α-hydroxy-7-oxoazorellano (azorellanone). Its molecular formula is $C_{20}H_{32}O_2$, and it corresponds to a tricyclic diterpene. Previous studies have indicated that cyclic terpene compounds such as triptolide decreased sperm motility and concentration in the rat (Lue et al, 1998; Sinha Hikim et al, 2000). In this work, we present the result of the isolation of the diterpenoid azo-

This work was financed by PROIM 1363 and Fondecyt 1011068.

Correspondence to: Dr Patricio Morales, Unit of Reproductive Biology, Faculty of Health Sciences, University of Antofagasta, PO Box 170, Antofagasta, Chile (e-mail: pmorales@uantof.cl).

Received for publication November 19, 2002; accepted for publication January 16, 2003.

rellanone from *A yareta* and its effect on several parameters that characterize human sperm function (ie, sperm motility and viability, sperm binding to the human zona pellucida, progesterone-induced acrosome reaction, and increase in intracellular Ca^{2+} concentration—$[Ca^{2+}]_i$) and protease activity.

Materials and Methods

Reagents

Progesterone, fura 2AM, Hoescht 33258, ethyl acetate, dimethylsulfoxide (DMSO), bovine serum albumin (BSA), petrol, silica gel, digitonin, and ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Sigma Chemical Company (St Louis, Mo). Succinyl-Leu-Leu-Val-Tyr 7-Amido-4-methylcoumaryn (Suc-LLVY-AMC) and N-t-Butyloxy-carbonyl-L-Gln-Ala-Arg 7-Amido-4-methylcoumaryn (Boc-GAA-AMC) were obtained from Affiniti Research Products Ltd (Exeter, United Kingdom). *Pisum sativum* agglutinin (PSA)-fluorescein isothiocyanate (FITC) was purchased from Vector Laboratories Inc (Burlingame, Calif).

Extraction and Isolation of the Azorellanone

A yareta Hauman plants were collected in January 1999 in ''Quebrada de las Llaretas'' in Vallenar, Chile (Loyola et al, 2001). A voucher specimen was deposited in the Herbarium of the Universidad de Concepción. The dried and finely powdered whole plant of *A yareta* Hauman (3.0 kg) was extracted with petrol at room temperature to give a gum (220 g). This extract was chromatographed by flash chromatography on Si gel (Loyola et al, 2001). Fraction 5, eluted with petrol-ethyl acetate (12:8) (23 g), was further purified on an Si gel column and eluted with petrol-ethyl acetate (14:6) to yield azorellanone (3.2 g). To test the effect of azorellanone on sperm, it was diluted in 0.5% ethyl acetate (vol/vol) at 0.015, 0.15, 1.5, and 3 mM.

Semen Samples

Semen samples were obtained after 2–3 days of sexual abstinence. All donors signed a form consenting to the use of their sperm cells for research purposes. All samples used were normal, according to World Health Organization (WHO) guidelines (1999). Motile sperm were separated using a double Percoll gradient, as described previously (Morales et al, 2000). Briefly, aliquots of semen were deposited on the upper Percoll layer and centrifuged at 300 × *g* for 20 minutes. The pellet was resuspended in 10 mL of modified Tyrode medium consisting of 117.5 mM NaCl, 0.3 mM NaH_2PO_4, 8.6 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 mM glucose, 0.25 mM sodium pyruvate, 19 mM sodium lactate, 70 μg/mL penicillin and streptomycin, phenol red, and 0.3% BSA (Suarez et al, 1986); was centrifuged again at 300 × *g* for 10 minutes; and finally, was resuspended in the same medium but supplemented with 2.6% BSA. The sperm concentration was adjusted to 10×10^6 cells/mL, and the suspension was incubated at 37°C with 5% CO_2 in air for 4.5 or 20 hours to promote capacitation.

Zona Pellucida Collection

Human oocytes were dissected from ovarian tissue obtained from cadavers and stored at −80°C as previously described (Overstreet et al, 1980; Cross et al, 1988). After thawing, the oocytes were freed of remaining cumulus cells by passing them through a narrow-bore pipette. As a result of freezing and thawing, these oocytes were not viable.

Evaluation of Sperm Motility and Viability

Sperm aliquots (400 μL) were incubated with different concentrations of azorellanone for 30 and 60 minutes at 37°C with 5% CO_2 in air. Control sperm were incubated with 0.5% ethyl acetate (vol/vol). The motility of the sperm was evaluated according to WHO guidelines (1999). Sperm viability was assessed by labeling the sperm with the supravital dye Hoescht 33258 and examining them with a fluorescence microscope. Results were expressed as the percentage of motile and alive sperm.

Sperm–Zona Pellucida Binding Assay

The sperm capacity to bind to the zona pellucida was evaluated using the hemizona assay (Burkman et al, 1988; Franken et al, 1989). Briefly, 49-μL droplets of 4.5-hour capacitated sperm were treated by adding 1 μL of test (azorellanone) or control solution (0.5% ethyl acetate [vol/vol]) under oil in a plastic petri dish. Then, 1 hemizona was added to the control sperm droplet, and the matching hemizona was added to the test sperm droplet. Control and test sperm droplets containing hemizonae were incubated for 10 minutes at 37°C with 5% CO_2 in air. After incubation, each hemizona was removed and gently washed with a wide-bore pipette. The tightly bound spermatozoa on the outer surface of each hemizona were counted under a phase-contrast microscope. These procedures have been described in detail elsewhere (Morales et al, 1994b, 1999; Morales, 1998).

Progesterone-Induced Acrosome Reactions

After the spermatozoa were incubated for 20 hours, some aliquots were incubated for 15 minutes with different concentrations of azorellanone. Control sperm were incubated with 0.5% ethyl acetate (vol/vol). Then, the sperm were treated for 15 minutes with 0.69 μM progesterone or its solvent (0.1% DMSO). Control sperm received 0.1% DMSO. Acrosomal status was evaluated using FITC-labeled PSA, as described (Cross et al, 1986; Morales et al, 1992).

Measurement of Intracellular Ca^{2+} Concentration

The progesterone-induced increase in $[Ca^{2+}]_i$ was evaluated as described (Garcia and Meizel, 1999; Morales et al, 2000). Briefly, 4.5-hour capacitated sperm were incubated with 3 μM of fura 2 for 30 minutes at 37°C with 5% CO_2 in air. The sperm were then washed in Tyrode medium with 2.6% BSA without phenol red and centrifuged twice at 300 × *g* for 5 minutes. Sperm aliquots were incubated with different concentrations of azorellanone for 15 minutes at 37°C with 5% CO_2 in air at a final concentration of $7–8 \times 10^6$ cells/mL. Control sperm were incubated with 0.5% ethyl acetate (vol/vol). Then, 1-mL sperm aliquots were used for spectrofluorometry, resuspending them directly into stirred fluorescence cuvettes. All these procedures were car-

ried out in the dark to prevent sample photobleaching. Fluorescence caused by $[Ca^{2+}]_i$ under various experimental conditions was monitored using a Shimadzu model 1501 (Kyoto, Japan) spectrofluorometer at an excitation wavelength pair of 340/380 nm and an emission wavelength of 510 nm. After equilibration for 2 minutes, measurements of $[Ca^{2+}]_i$ were started. Approximately 50 seconds after the beginning of each sample run, progesterone (0.69 μM) was added to the sperm suspension. Sequential additions of 20 μM digitonin and 10 mM Tris-EGTA were made near the end of each experiment to facilitate determination of $[Ca^{2+}]_i$, as previously described. To further analyze the results, the highest value of $[Ca^{2+}]_i$ (peak) for each treatment was obtained, and the area under the curve during the first 300 seconds of treatment was measured using a planimeter GTCO Quickruler (Columbia, Md) (Morales et al, 2002).

Preparation of Sperm Extracts

Crude sperm extracts to evaluate protease activity were obtained as described previously (Morales et al, 1994a, 2002). Briefly, sperm were separated from seminal plasma, other cell types, and cellular debris by centrifugation through a column of Percoll as described above except that the Percoll was prepared in 50 mM HEPES and 191 mM NaCl, pH 7.4 (Morales et al, 1994a). The resulting sperm pellet was washed 2 times by centrifugation at $300 \times g$ for 10 minutes and then resuspended in homogenization buffer (50 mM HEPES and 10% glycerol, pH 7.4) at a concentration of 25×10^7 sperm/mL. The sperm suspension was then sonicated (Virsonic, Gardiner, NY) with seven 60-W bursts of 30 seconds each and centrifuged for 30 seconds at $5000 \times g$ in a Beckman microfuge to remove nuclear and flagellar material. The supernatant was used as the enzyme stock preparation. All these procedures were performed at 4°C. The protein concentration in each sperm extract preparation, obtained using the Bradford method (Bradford, 1976), ranged between 0.3 and 0.8 mg/mL. The trypsin-like and chymotrypsin-like activities of the sperm extracts were assayed using the fluorogenic substrates Suc-LLVY-AMC and Boc-GAA-AMC. Aliquots of 100 μL of enzyme extract were incubated in a final volume of 2 mL containing 10 mM $CaCl_2$, 50 mM HEPES, pH 7.4, and 10 μM substrate. The assay was run at 37°C, and the fluorescence was monitored with excitation at 380 nm and emission at 460 in a Shimadzu model 1501 spectrofluorometer.

To test the effect of the azorellanone on the sperm proteolytic activity, 100-μL aliquots of the extract were preincubated with different concentrations of azorellanone for 15 minutes at 37°C before adding the substrates. Control sperm were incubated with 0.5% ethyl acetate (vol/vol). In some experiments, free-swimming sperm were incubated with azorellanone prior to preparation of the extracts.

Statistical Analysis

The data were analyzed by analysis of variance for repeated measures using the StatView program (SAS Institute, Cary, NC) on an Apple Power Macintosh 6500/225. Differences between individual groups were examined with Fisher's protected least significant difference test. Paired comparisons were conducted using a paired *t* test, and all data are presented as mean values plus or minus standard errors of the mean. Differences were considered significant at the .05 level of confidence.

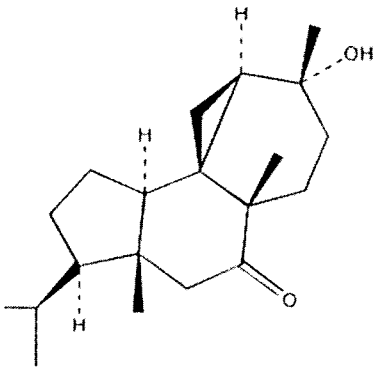

Figure 1. Chemical structure of the 13-α-hydroxy-7-oxoazorellano (azorellanone).

Results

Isolation and Structure of Azorellanone

Azorellanone was isolated as white crystals (melting point, 166°C–168°C). Its molecular formula was $C_{20}H_{32}O_2$, deduced by a combination of ^{13}C-nuclear magnetic resonance (NMR) data and high-resolution mass spectrometry, which yielded a parent ion of $[M\text{-}H_2O]^+$ (obtained 286.2143 atomic mass units; required 286.2297 atomic mass units). ^{13}C-NMR, DEPT (distortion less enhancement by polarization transfer), and ^1H-NMR showed 5 degrees of unsaturation (Figure 1). Its infrared spectrum suggested the presence of 1 OH^- group and 1 carbonyl acetate group. Therefore, the structure of azorellanone corresponds to a tricyclic diterpene. The analysis of the ^1H-NMR and ^{13}C-NMR spectra for azorellanone suggested that this compound corresponded to 13-α-hydroxy-7-oxoazorellano. The proposed structure was confirmed by the transformation of known 7-desacetylazorellanol 1 into 13-α-hydroxy-7-oxoazorellano (azorellanone) by means of oxidation with pyridium chlorochromate. Table 1 shows the data for the NMR ^1H and NMR ^{13}C spectra of azorellanone.

Biological Effect of Azorellanone

Regarding sperm motility, treatment with azorellanone significantly reduced the number of free-swimming sperm. This effect was observed starting at 0.15 mM and was concentration-dependent (Table 2). There was no further decline in motility when the cells were incubated for 60 minutes instead of 30 minutes. This effect of azorellanone on sperm motility, however, was not strictly associated with a decrease in sperm viability. In effect, the concentration of azorellanone necessary to decrease the number of living sperm was higher than that required to reduce sperm motility (Table 2).

Table 1. *^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectroscopic data of compound 13-α-hydroxy-7-oxoazorellano (azorellanone)*†*

Nº C/H	δ_C‡	δ_H§	ROESY	HMBC
1	21.1 t	β 1.35 α 0.98 dd (6, 12)	. . .	. . .
2	27.3 t	β 1.84 m ax α 1.22 dd eq		2,3,5
3	60.6 d	1.20		
4	31.3 d	1.50 m		2,3,5
5	43.5 s			
6	53.5 t	β 2.75 d (17.9) eq α 2.55 d (17.9) ax	17,20	7
7	218.0 s			
8	44.9 s			
9	26.9 s			
10	52.4 d	α 2.00 dd (7.6, 11.6)	6α	1,2,3,6,11,20
11	10.0 t	β 0.23 t eq (6.2) α 0.92	17,16	
12	24.9 d	0.75 dd (6.2, 10.0)		9,10,11,13,14
13	69.2 s			
14	27.04 t	β 1.18 α 1.45 m ax		8,13,16
15	30.8 t	β 1.20 α 1.76		7,8,13,14
16	30.1 q		11β	12,13,14
17	25.9 q			7
18	22.7 q‖	0.92 d (6.5)		3,4
19	22.4 q‖	0.84 d (6.5)		3,4
20	16.3 q	1.03 s	18,19	3,6,10

* HMBC indicates heteronuclear multibond connectivity; NMR, nuclear magnetic resonance; Nº C/H, number of C/H atoms in the molecule; and ROESY, rotating-frame Overhauser enhancement spectroscopy.

† Spectra take in $CDCl_3$.

‡ Multiplicity determined from DEPT (distortionless enhancement by polarization transfer).

§ Coupling constants (J in Hz) in parentheses.

‖ Plus sign indicates interchangeability.

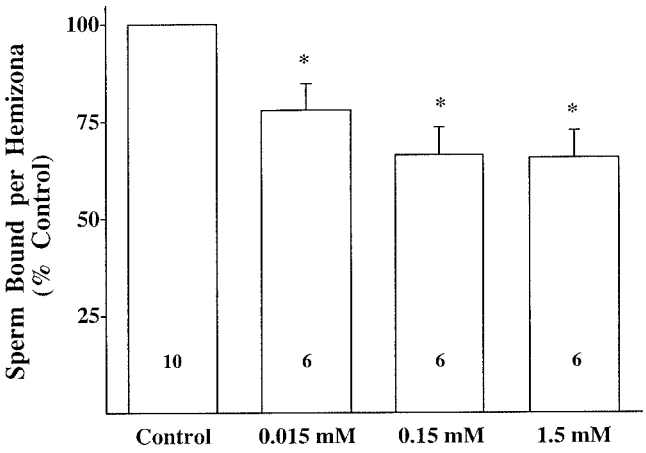

Figure 2. Effect of 13-α-hydroxy-7-oxoazorellano (azorellanone) on the number of spermatozoa bound to the human hemizona pellucida. Data are expressed as a percentage (mean ± SEM) of the control hemizonae. Sperm suspensions were incubated for 5 minutes with **(a)** saline (control) or different concentrations of azorellanone prior to the hemizona assay. The figure within the bars indicates the number of experiments in each group. * Significantly different from the control group ($P = .0015$).

With regard to the sperm's ability to bind to the human zona pellucida, the results indicated that azorellanone significantly inhibited sperm–zona pellucida binding at all concentrations tested (Figure 2), including the concentration of 0.015 mM, which did not affect sperm movement. In addition, treatment with 1.5 mM azorellanone significantly inhibited the occurrence of the acrosome reaction stimulated by progesterone (Figure 3). Thus, while the percentage of acrosome-reacted sperm rose from 18.6% plus or minus 4.4% (control) to 41.4% plus or minus 6.1% after only progesterone treatment ($P < .001$), in the sperm previously treated with 1.5 mM azorellanone, the percentage of acrosome-reacted sperm after progesterone treatment was only 16.2% plus or minus 2.2%.

With regard to the progesterone-induced increase in $[Ca^{2+}]_i$, this was about 3 times the basal value (Figure 4A). The basal Ca^{2+} levels were the same whether the sperm were treated with 0.1 DMSO or 1.5 mM azorellanone (data not shown). Thus, progesterone treatment alone increased the intracellular Ca^{2+} concentration from 317 plus or minus 25 nM to 993 plus or minus 63 nM. However, previous treatment of the sperm with azorellanone significantly inhibited the maximum value reached in the intracellular Ca^{2+} concentration stimulated by progesterone, where azorellanone concentrations were 0.15 and 1.5 mM. The $[Ca^{2+}]$ levels were 503 ± 48 nM and 683

Table 2. *In vitro effect of 13-α-hydroxy-7-oxoazorellano (axorellanone) on human sperm motility and viability*

	Concentration of Azorellanone (mM)*									
	0		0.015		0.15		1.5		3.0	
Time (min)	30	60	30	60	30	60	30	60	30	60
% Motile cells	85 ± 2	81 ± 3	79 ± 2	72 ± 3	65 ± 6†	68 ± 3†	61 ± 2†	61 ± 3†	44 ± 7†	41 ± 6†
% Living cells	89 ± 2	85 ± 2	89 ± 1	89 ± 1	84 ± 1	74 ± 3	73 ± 5	73 ± 7	72 ± 4	57 ± 12†

* Sperm aliquots were incubated with different concentrations of azorellanone for 30 and 60 minutes. The percentage of motile sperm and their viability were then evaluated. The results represent the average, plus or minus the standard error of the mean, of 6 duplicate experiments conducted with 5 different semen samples.

† Significantly different from the control ($P < .01$).

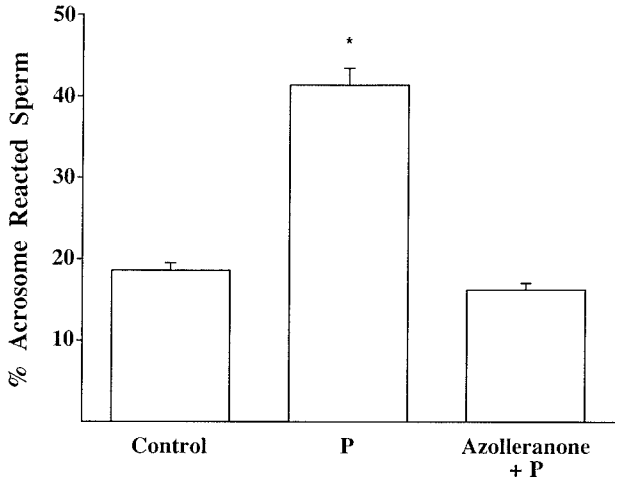

Figure 3. Effect of 13-α-hydroxy-7-oxoazorellano (azorellanone) on the progesterone-induced sperm acrosomal exocytosis. Sperm aliquots were treated with 0.1% dimethylsulfoxide (DMSO) (control), progesterone (P, 0.69 μM), or azorellanone (1.5 mM) and then progesterone (azorellanone + P). Results are the mean plus or minus the standard deviation of 6 experiments conducted with 5 different semen samples. * Significantly different from the other groups ($P = .0015$).

± 58 nM, respectively. This was also evident when the area under the Ca^{2+} peak was analyzed (Figure 4B).

Finally, with regard to the effect of azorellanone on the protease activity of human sperm extracts, incubation with azorellanone significantly inhibited both the trypsin-like (Figure 5A) and the chymotrypsin-like activity of the extracts (Figure 5B). The specific activity toward both trypsin and chymotrypsin substrates was significantly inhibited in a concentration-dependent manner (Table 3). The inhibition of protease activities was observed only when the sperm extracts were incubated with azorellanone; incubation of free-swimming sperm with azorellanone did not inhibit the protease activity of the extracts (data not shown).

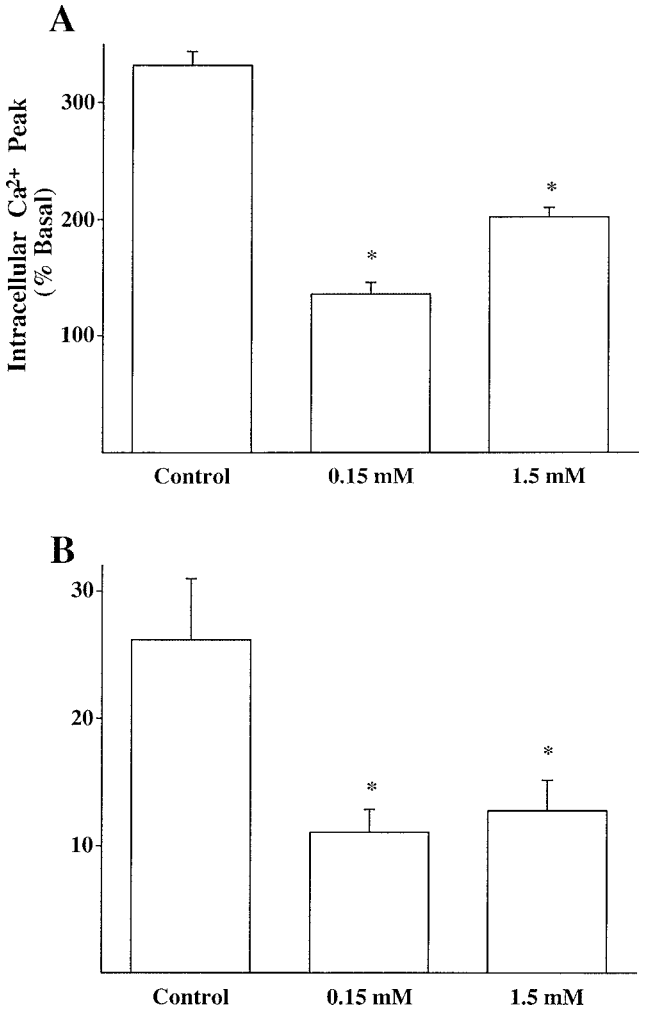

Figure 4. Effect of 13-α-hydroxy-7-oxoazorellano (azorellanone) on the progesterone-induced increase in $[Ca^{2+}]_i$. **(Panel A)** The mean plus or minus the standard deviation (n = 6) of the peak progesterone-induced increase in $[Ca^{2+}]_i$ is shown. Data are expressed as a percentage of the basal value. **(Panel B)** The area under the curve (mm2) for the progesterone-induced increase in $[Ca^{2+}]_i$ is shown. * Significantly different from the progesterone value ($P < .001$).

Discussion

Natural compounds of vegetal origin have been tested for many years for their capacity to inhibit male fertility. In this study, we present evidence that suggests that a diterpene of an azorellane type, namely 13-α-hydroxy-7-oxoazorellano (azorellanone), extracted from the high Andes shrub *A yareta* inhibited several parameters that characterize human sperm function. In effect, treatment of human sperm with azorellanone inhibited 1) sperm motility without significantly affecting sperm viability; 2) sperm–zona pellucida binding; progesterone-induced acrosomal exocytosis and an increase in $[Ca^{2+}]_i$; and 3) trypsin-like and chymotrypsin-like sperm protease activities.

With regard to sperm motility and viability, the number of motile sperm was significantly reduced with azorellanone in a concentration-dependent manner, while the latter was affected only at the highest concentration tested (3 mM). The mechanism by which azorellanone decreased flagellar beat is not clear. However, the work of Huynh et al (2000) with triptolide demonstrated that this diterpene extracted from the Chinese medicinal plant *T wilfordii* inhibited the motility of rat epididymal sperm. In addition, electron microscopy studies suggested that triptolide provoked severe alterations in the sperm ultrastructure, the most conspicuous being the absence of a sperm plasma membrane over the middle and principal piece of the flagellum and premature decondensation of the nuclei (Sinha Hikim et al, 2000). The loss of the plasma membrane from these sperm cells explained their loss of motility. Therefore, the viability of the sperm was also af-

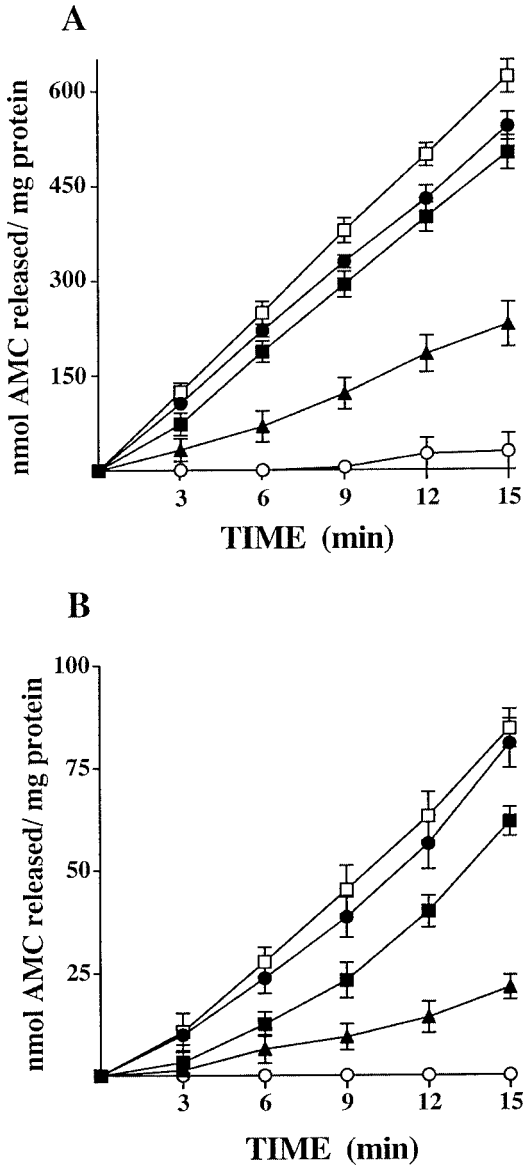

Figure 5. Effect of 13-α-hydroxy-7-oxoazorellano (azorellanone) on the trypsin-like **(A)** and chymotrypsin-like **(B)** activities of human sperm. Sperm extracts were incubated with solvent (open squares) or with 0.015 mM (dark circles), 0.15 mM (dark squares), 1.5 mM (triangles), or 3 mM (open circles) azorellanone. Then, the synthetic trypsin (Boc-GAA-AMC) or chymotrypsin substrates (Suc-Leu-Leu-Val-Tyr-AMC) were added. Results are the mean plus or minus standard error of the mean of 6 experiments conducted with 5 different semen samples.

fected in the same manner as motility. According to this, azorellanone could be a potential contraceptive agent, since mammalian sperm must use their flagellar apparatus to move along the female genital tract, from their site of deposition in the vagina to the site of fertilization in the ampulla of the oviduct (Yanagimachi, 1994). In addition, it is highly unlikely that immotile mammalian sperm could traverse the zona pellucida (van Kooij et al, 1985; Mellinger and Goldstein, 1987; Bedford, 1998).

We also showed in this study that azorellanone inhibited the sperm's ability to bind to the human zona pellucida. This inhibition by azorellanone cannot be attributed only to a decrease in sperm motility after treatment, because the effect was also caused by an azorellanone dose of 0.015 mM, which did not affect sperm motility. Perhaps more subtle alterations of the sperm plasma membrane could explain this result.

Azorellanone inhibited the progesterone-induced acrosomal exocytosis and increase in $[Ca^{2+}]_i$. It has been shown that plasma membrane molecules in mammalian sperm are important for the sperm's migration to the site of fertilization and that the functional integrity of the plasma membrane is an important factor in sperm motility, sperm acrosome reaction, capacitation, metabolism, and binding to the zona pellucida (Fraser and Ahuja, 1988). Therefore, it is possible that azorellanone may be altering a surface molecule(s) responsible for the effects mentioned above. Similar changes in the plasma membrane could be involved in altering its ionic conductance, in this case to Ca^{2+}. The latter could also be related to changes in the flagellar movement (Porat, 1990).

With regard to the inhibition of the trypsin-like and chymotrypsin-like protease activities of the sperm, azorellanone inhibited both activities in a concentration-dependent manner. This was the case when sperm extracts were incubated with azorellanone. Thus, this could be an additional mechanism by which azorellanone impaired sperm function. Other plant extracts such as gossypol also inhibited the activity of certain enzymes that are involved in the metabolic regulation of the sperm (Maugh, 1981; Nakamura et al, 1988; Rajpurohit and Giridharan, 1988). Moreover, gossypol inhibition of the serine protease acrosin (EC 3.4.21.10, an enzyme of acrosomal origin with trypsin-like activity) in pigs has also been suggested as a

Table 3. *Effect of 13-α-hydroxy-7-oxoazorellano (azorellanone) on the trypsin-like and chymotrypsin-like protease activities of human sperm extracts*

	Concentration of Azorellanone (mM)				
Protease activity*	0	0.015	0.15	1.5	3.0
Trypsin-like	33.6 ± 2.1	40.5 ± 3.5†	27.9 ± 3.2†	13.4 ± 4.6†	1.6 ± 1.0†
Chymotrypsin-like	4.4 ± 0.8	4.8 ± 0.9†	2.6 ± 0.6†	1.0 ± 0.2†	0 ± 0†

* The protease activity is expressed in nmol Amido-4-methylcoumaryn hydrolyzed/mg protein/min.
† Significantly different from its respective control ($P < .0001$).

mechanism of sperm function impairment (Sadykov et al, 1985). The scanty or nil effect of azorellanone on the enzyme activity when added to whole sperm did not necessarily imply that this compound did not have an effect on the enzyme activity. Damage to the plasma membrane could be a first event in the interaction between azorellanone and the sperm, and then azorellanone would enter the cytoplasm, inhibiting its protease activities. Experiments with longer incubation times than the ones used in this study may confirm this hypothesis.

In summary, the plant origin compound azorellanone significantly inhibited several functions that are essential for fertilization, such as sperm motility, sperm–zona binding, sperm acrosome reaction and Ca^{2+} influx, and sperm protease activities. Azorellanone had a spermicidal effect only at the highest dose tested (3 mM). All these results allow us to think that azorellanone may be a potential candidate as a contraceptive agent to be used in the manufacture of vaginal jellies or creams. Experiments are under way to determine the in vivo effect of azorellanone.

References

Bedford JM. Mammalian fertilization misread? Sperm penetration of the eutherian zona pellucida is unlikely to be a lytic event. *Biol Reprod.* 1998;59:1275–1287.

Bradford M. Rapid and sensitive methods for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem.* 1976;72:248–254.

Burkman LJ, Coddington CC, Franken DR, Kruger TF, Rosenwaks Z, Hodgen GD. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril.* 1988;49:688–697.

Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome reacted human sperm. *Gamete Res.* 1986;15: 213–226.

Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of the acrosome reaction by human zona pellucida. *Biol Reprod.* 1988;38:235–244.

Franken DR, Oehninger SC, Burkman LJ, Coddington CC, Kruger TF, Rosenwaks Z, Acosta AA, Hodgen GD. The hemizona assay (HZA): a predictor of human sperm fertilizing potential in in vitro fertilization (IVF) treatment. *J. In Vitro Fert Embryo Transfer*. 1989;6:44–50.

Fraser LR, Ahuja KK. Metabolic and surface events in fertilization. *Gamete Res.* 1988;20:491–519.

Garcia MA, Meizel S. Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: pharmacological investigation of T-type calcium channels. *Biol Reprod.* 1999;60:102–109.

Huynh PN, Sinha Hikim AP, Wang C, et al. Long-term effects of triptolide on spermatogenesis, epididymal sperm function, and fertility in male rats. *J Androl.* 2000;21:689–699.

Loyola LA, Bórquez J, Morales G, Neira I, Araya J, González J, Sagua H, San Martín A. Diterpenoids from *Azorella yareta* and their trichomonacidal activities. *Phytochemistry.* 2001;56:177–180.

Loyola LA, Bórquez J, Morales G, San Martín A. Mulinol, a diterpenoid from *Azorella compacta. Phytochemistry.* 1997;45:1465–1467.

Loyola LA, Bórquez J, Morales G, San Martín A. 11,12-epoxi-mulin-11-en-20-oico, a diterpenoid from *Azorella compacta. Phytochemistry*. 1998a;49:1091–1093.

Loyola LA, Bórquez J, Morales G, San Martín A, Manriquez V, Wittke O. Azorellanol: a diterpenoid with a new carbon skeleton from *Azorella compacta. Tetrahedron.* 1998b;54:15533–15540.

Lue Y, Sinha Hikim AP, Wang C, et al. Triptolide: a potential male contraceptive. *J Androl.* 1998;19:479–486.

Maugh TH. Male ''pill'' blocks sperm enzyme. *Science.* 1981;212:314.

Mellinger B, Goldstein M. New areas of research in male infertility. *Urol Clin North Am.* 1987;14:619–632.

Morales P. Gonadotropin-releasing hormone increases ability of the spermatozoa to bind to the human zona pellucida. *Biol Reprod.* 1998;59: 426–430.

Morales P, Llanos M, Gutierrez G, Kohen P, Vigil P, Vantman D. The acrosome reaction inducing activity of individual human follicular fluid samples is highly variable and is related to the steroid content. *Hum Reprod.* 1992;7:646–651.

Morales P, Pizarro E, Kong M, Kerr B, Ceric F, Vigil P. Gonadotropin-releasing hormone-stimulated sperm binding to the human zona is mediated by a calcium influx. *Biol Reprod.* 2000;63:635–642.

Morales P, Pizarro E, Kong M, Pasten C. Sperm binding to the human zona pellucida and calcium influx in response to GnRH and progesterone. *Andrologia.* 2002;34:1–7.

Morales P, Socias T, Cortez J, Llanos MN. Evidences for the presence of a chymotrypsin-like activity in human spermatozoa with a role in the acrosome reaction. *Mol Reprod Dev.* 1994a;38:222–230.

Morales P, Vantman D, Madariaga M. Sperm penetration through the human zona pellucida as a predictor of in vitro fertilization. *Andrologia.* 1999;31:131–135.

Morales P, Vigil P, Franken DR, Kaskar K, Coetzee K, Kruger TF. Sperm–oocyte interaction: studies on the kinetics of zona pellucida binding and acrosome reaction of human spermatozoa. *Andrologia.* 1994b;26: 131–137.

Nakamura M, Ideda M, Susuki A, Okinaga S, Arai K. Metabolism of round spermatids: gossypol induces uncoupling of respiratory chain and oxidative phosphorylation. *Biol Reprod.* 1988;39:771–778.

National Coordinating Group on Male Antifertility Agents. Gossypol: a new antifertility agent for males. *Chin Med J.* 1978;4:417–428.

Overstreet JW, Yanagimachi R, Katz DF, Hayashi K, Hanson FW. Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster eggs: a study of fertile donors and infertile patients. *Fertil Steril.* 1980;33:534–554.

Ponce-Monter H, Estrada AV, Pedron N, Valencia A, Gallegos AJ. The zoapatle. X. The in vitro effect of zoapatle aqueous crude extract (ZACE) and histamine upon rat guinea pig uterine strips. *Contraception.* 1985;31:533–541.

Porat O. Effects of gossypol on the motility of mammalian sperm. *Mol Reprod Dev.* 1990;25:400–408.

Rajpurohit R, Giridharan N. Failure of oral gossypol to inhibit hepatic microsomal and cytosolic drug-metabolising enzymes. *Biochem Pharmacol.* 1988;37:4590–4592.

Sadykov AS, Ismailov AI, Zakhidov ST, Baram NI. Effect of gossypol on spermatogenesis and fertility in man and mammals. *Ontogenez.* 1985;16:346–357.

Sinha Hikim AP, Lue YH, Wang C, Reutrakul V, Sangsuwan R, Swerdloff RS. Posttesticular antifertility action of triptolide in the male rat: evidence for severe impairment of cauda epididymal sperm ultrastructure. *J Androl.* 2000;21:431–437.

Suarez SS, Wolf DP, Meizel S. Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. *Gamete Res.* 1986;14:107–121.

Van Kooij RJ, Balerna M, Campana A. Biological aspects of in vitro fertilization. *Experientia.* 1985;41:1496–1502.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interactions.* 4th ed. Cambridge, United Kingdom: Cambridge University Press; 1999:138.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* New York, NY: Raven Press; 1994: 189–317.