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IN VITRO EFFECTS OF ARECOLINE ON SPERM MOTILITY AND CYCLOOXYGENASE-2 EXPRESSION

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ABSTRACT — Semen samples were obtained from 30 volunteers who had never consumed betel quid. Swim-up spermatozoa from the 30 seminal samples of non-betel quid chewers and also non-smokers, usually not exposed to passive smoking, were treated *in vitro* with arecoline at different concentrations to evaluate the action of these drugs on sperm motility. Highly motile sperms were collected and divided into 5 equal fractions. Four fractions were supplemented with various concentrations of arecoline and one as control. The study was carried out at time 0 and +1, +2, +3 and +4 hr of incubation. Sperm cells were also extracted and blotted with COX-2 antibody after arecoline treatment after 4 hr incubation. The sperm motility parameters, i.e., motility, average path velocity, curvilinear velocity, straight-line velocity and linearity, were significantly decreased after arecoline treatment. *In vitro*, arecoline induces the COX-2 expression of sperm cells in a dose-dependent manner. This is the first report to demonstrate that arecoline may mediate COX-2 expression in human sperms, resulting in inflammation response. This situation may act on the structure responsible for the flagellar motion and cause the reduction of sperm motility.

KEY WORDS: Arecoline, Human sperm, Motility, CASA, COX-2

INTRODUCTION

It has been estimated that there are 200-600 million people who have the betel quid (*Areca catechu* L.) chewing habit during their lifetime (Burton, 1979; Sharan, 1996; IARC, 1985). In Taiwan, it is estimated that two million people chew it habitually (Ko *et al*., 1992). Betel quid chewing is strongly associated with oral submucous fibrosis, leukoplakia, and cancer (IARC, 1985; Thomas *et al*., 1993; Ko *et al*., 1995; Jacob *et al*., 2004). Many of the undesirable effects of betel quid have been attributed to arecoline, the major alkaloid of betel nuts, demonstrating mutagenicity, genotoxicity and cytotoxicity in various mammalian cells (Sharan, 1996; Sundqvist *et al*., 1989; Jeng *et al*., 1999; Chang *et al*., 2001; Jeng *et al*., 2001). For example, arecoline (0.4-1.2 mM) increased hyperpolarization of mitochondrial membrane potential and induced little DNA fragmentation on KB cells within 24 hr (Chang *et al*., 2001).

In male mice, arecoline has the ability to change gonad morphofunction, including shape abnormality of sperm (Sinha and Rao, 1985) and unscheduled DNA synthesis in germ cells and other human cells (Sinha and Rao, 1985; Sharan and Wary, 1992). Using Chinese hamster ovary cells, arecoline yielded a dose-dependent increase in the frequencies of sister-chromatid exchanges and chromosomal aberrations (Dave *et al*., 1992), and in the number of micronucleated cells (Lee *et al*., 1996).

The association of betel quid chewing and the expression of cyclooxygenase-2 (COX-2), an inducible rate-limiting enzyme in prostaglandin synthesis, has been documented to indicate that areca nut ingredients were involved in the pathogenesis of oral submucous fibrosis and oral cancer (Jeng *et al*., 2000). In male mouse gonad, microsomal PGE synthase-1 was detected in Leydig cells of the testis and in epithelial cells of the epididymis, vas deferens, and seminal vesicle. Meanwhile, COX-2 was dominantly expressed in the vas deferens and the epithelial cells of the distal cauda epididymis (Lazarus *et al*., 2002). Prominent

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COX-2 expression was also found in the distal vas deferens of the rat (MdKanna *et al*., 1998). On the contrary, only low levels of COX-2 were found in human testes (O'Neill *et al*., 1993). Functionally, the opposite effect of PGE (stimulation) and PGF (suppression) on the regulation of sperm functions was also noted (Gottlieb *et al*., 1988). Unfortunately, no information is available on the association between arecoline and human sperm function. The present study was undertaken to examine whether sperm functions are damaged in chronic betel quid chewers and to explore the possible mechanism, if any, via the COX-2 pathway.

MATERIALS AND METHODS

Collection and preparation of normospermic samples

Semen samples were collected from 30 healthy men who did not chew betel quid (mean age: 24.67±4.8 years) after 4 days of sexual abstinence. After seminal liquefaction, those samples were subjected for analyses using a Hamilton-Thorn Motility Analyzer (version10.9i; Hamilton Throne Research, Beverly, MA). The settings used for analysis were as follows: frame rate, 60Hz; frames acquired, 30; minimum contrast, 80; minimum cell size, 3 pixels; threshold straightness, 80%; low-size gate, 1; high-size gate, 2.9; low-intensity, 0.6; high-intensity, 1.4 and magnification factor, 0.95.

The motility parameters including rapid progressive motility (i.e., actual space-gain motility), average path velocity (i.e., the average velocity of sperm movement exhibiting rapid progressive motility in an average path velocity more than 25 μm/s), straight-line velocity (VSL, the straight-line distance from beginning to end of a sperm track divided by the time taken to travel that distance), curvilinear velocity (VCL, a measure of the total distance traveled by a given sperm divided by the time elapsed), the amplitude of lateral head displacement (ALH, the mean width of sperm head oscillation), beat cross-frequency (BCF, the frequency with which the sperm head crosses the sperm average path), straightness (STR, ratio of VSL/VCL), elongation (ratio of minor to major axis of sperm head), and area (area of head size). All of the volunteers provided written informed consent and were approved by the hospital IRB committee.

Only those normospermic samples were eligible for study. Normospermia were defined by World Health Organization criteria, i.e., volume ≥ 2.0 ml, sperm concentration ≥ 20×10^6 spermatozoa/ml, motility ≥ 50%, normal morphology ≥ 30%, and < 1×10^6 leukocytes/ml. For study, those samples were diluted at 1:1 (v/v) with 10% fetal cord serum supplemented with Ham's F-10 culture medium (Gibco BRL, Grand Island, NY) and washed twice by centrifugation (300 g, 5 min). Highly motile sperm populations were collected by allowing sperm pellets to swim-up during incubation at 37°C for 1 hr and were then immediately moved for further study.

Determination of sperm motility parameters

Samples were divided into 5 equal fractions. Various concentrations of arecoline (0, 10, 50, 100, and 200 μg/ml in HAM-F10 medium) were added to each fraction and fractions were incubated at 37°C under 5% CO_2. High doses of arecoline were prepared to evaluate sperm reaction to massive doses of these substances. Such high levels of arecoline may not have been found in semen. Measurements of sperm motility parameters from each fraction were analyzed at 0, 1, 2, 3, and 4 hr, respectively. Sperm motility parameters were measured at 37°C as described above.

SDS-PAGE and COX-2 immunoblotting

Immunoblotting procedures were performed as described previously (16). Equal amounts of sperm lysates (25 μg/lane), after treatment with different concentrations of arecoiline (0, 10, 50, 100, and 200 μg/ml) for 4 hr at 37°C, were separated by 10% polyacrylamide gel and transferred to nitrocellulose membrane with a semi-dry electroblotting apparatus (TE series Transphor Electrophoresis Unit, Hoefer Scientific Instruments, San Francisco, CA). The membrane was blocked overnight at room temperature with blocking reagent (100ml X10 TBS, 0.1%Tween 20, 50g non-fat dry milk and 1 g NaN_3) and incubated for 24 h by gentle shaking with mouse anti-human COX-2 monoclonal antibody (Santa Cruz Biotechnology Inc., CA) at 1:500 dilution of blocking buffer. Membrane was washed three times and incubated with peroxidase-linked species-specific sheep anti-mouse antibody (Santa Cruz Biotechnology Inc., CA). The protein expression of β-actin, as a marker for protein loading, was determined by using a mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology Inc., CA) at 1:5000 dilutions.

The bands were detected by chemiluminescence ECL kit (Pierce, Rockford, IL). Meanwhile, a computerized digital imaging system using Alphalmage 2200 software (Alpha Innotech, San Leandro, CA) was applied to examine the COX-2 protein to β-actin ratio of the bands obtained from the Western blot analysis.

Statistical analysis

Student's *t*-test was used for statistic analyses and $p<0.05$ was considered as statistically significant. Western blotting assay was performed three times to ensure reproducibility.

RESULTS

Effect of arecoline on sperm motility *in vitro*

Treatment of arecoline caused a significant reduction of motile sperm as compared with that in the control group in a dose-dependent manner (Fig. 1A). Sperm motility was inhibited by 18.4%, and 49.6% in the presence of arecoline at 100 and 200 μg/ml after 2 hr of incubation, respectively, ($p<0.01$). After 4 hours of treatment, significant decreases of 16.3%, 57% and 95.4% were observed with arecoline concentrations at 50, 100 and 200 μg/ml, respectively ($p< 0.01$). Low-dose of arecoline concentrations (1 ng/ml-1000 ng/ml) were also added into sperm fractions. However, there

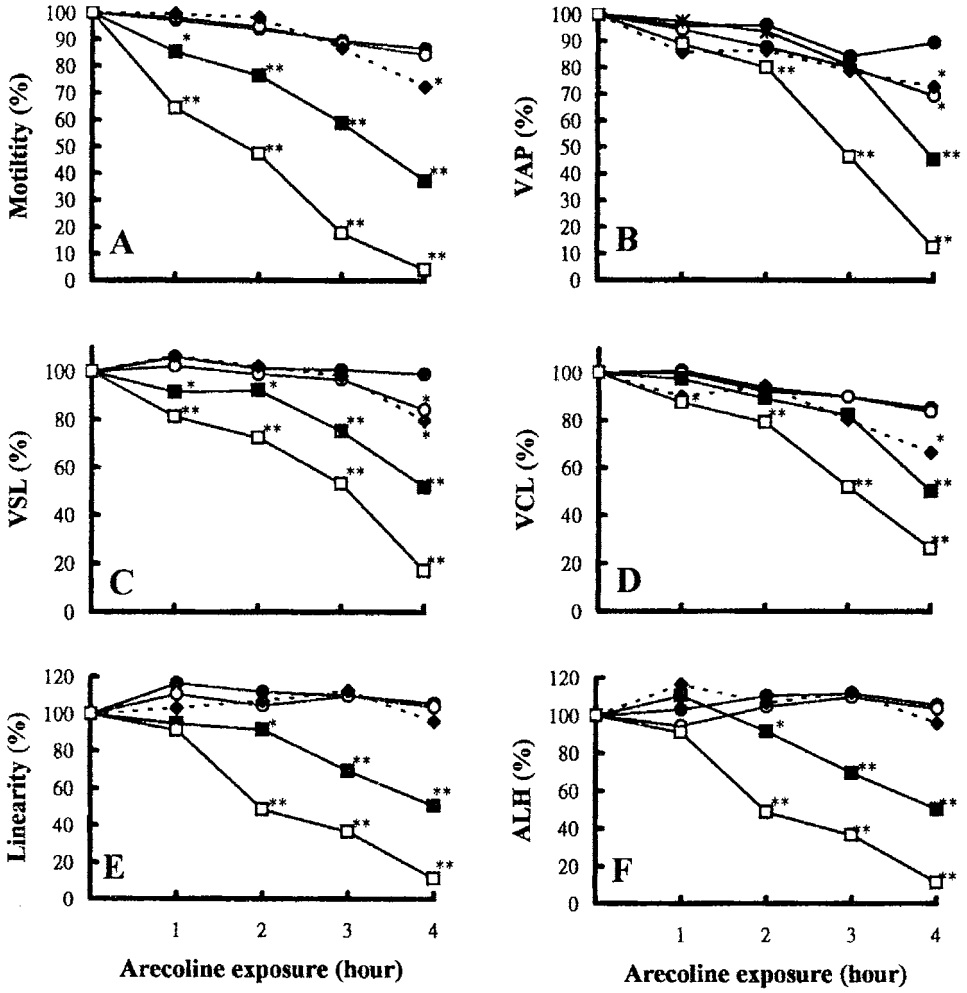

Fig. 1. The effect of arecoline *in vitro* on the motility parameters of human sperm (n=30) over a 4-hour period. Value change in mean percentage as compared with the contro —●—) at each point observed. Data show changes in percentage after incubation with arecoline in concentrations of 10 μg/ml (—○—), 50 μg/ml (····◆····), 100 vg/ml (—■—) and 200 μg/ml (—□—). (A), Motility; (B), Smoothed path velocity (VAP); (C), Straight-line velocity (VSL); (D), Curvilinear velocity (VCL); (E), Linearity (LIN) and (F), Amplitude of lateral head displacement (ALH).

* indicating $p<0.05$ and ** indicating $p<0.001$.

was no significant reduction in sperm motility (data not shown).

Effect of arecoline on sperm velocity *in vitro*

There was a significant reduction in smoothed path velocity (VAP) (Fig. 1B), straight-line velocity (VSL) (Fig. 1C) and curvilinear velocity (VCL) (Fig. 1D) after arecoline treatment. However, after 4 hr incubation, there was a significant decrease of VAP by 18.6%, 22.4%, 49% and 86% (Fig. 1B), of VSL by 14.8%, 19.5%, 47.6% and 83% (Fig. 1C) at the concentrations of 10, 50, 100 and 200 μg/ml arecoline, respectively, ($p<0.05$). VCL was inhibited by 21.8%, 41% and 69.2% (Fig. 1D) at the concentrations of 50, 100, 200 μg/ml arecoline, respectively, ($p<0.05$).

Effect of arecoline on linearity and amplitude of lateral head displacement of sperm *in vitro*

Arecoline exposure caused a significant reduction in LIN ($p<0.01$) in the presence of concentrations with 100 and 500 μg/ml after 3 and 4 hr of incubation (Fig. 1E). However, there were no significant changes in the dosages of 10 and 50 μg/ml. ALH decreased significantly by 52.2% and 89.4% in the presence of 100 and 200 μg/ml arecoline, respectively, after 4 hr of incubation ($p<0.01$) (Fig. 1F).

DISCUSSION

Reports of arecoline in association with human reproduction are rare. There is probably only one that can show that betel quid chewing is associated with the risk of adverse birth outcomes of pregnant women in an epidemiologic survey (Yang *et al*., 2001). No information is available on the association, if any, between arecoline intake and the male reproductive system. In chronic betel quid chewers, arecoline-induced COX-2 production indicates the COX-2/PG inflammatory pathway involving in the oral cytotoxicity and tumorigenesis (Lee *et al*., 1996; Jeng *et al*., 2003; tsai *et al*., 2003). Other mechanisms of oral pathogenesis provoked by betel quid involve metalloproteinase-1 (Shieh *et al*., 2003), cytokines (Hsu *et al*., 2001) and c-jun protooncogene (Ho *et al*., 2000). It has been demonstrated that mPGES-1 is expressed coordinately with COX-1 and COX-2 and is involved in PGE2 production in male genital organs (Lazarus *et al*., 2002). However, no evidence is available on the correlation between arecoline and COX-2 pathway in sperm kinetics. Arecoline damages DNA synthesis of germ cells in mice (Sinha., 1985) and ovary cells of Chinese hamster (Lee *et al*., 1996) and also induces sperm shape abnormality (Sinha., 1985). Thus, it is reasonable to conduct the present study to investigate whether betel quid disturbs spermatozoa development and function in long-term chewers and, also to examine the possibility of whether COX-2 plays a kinetic role in human sperm cells as it does in the mouse.

The first rate-limiting step in the conversion of arachidonic acid to prostaglandins (PGs) is catalyzed by COX. Two isoforms of COX have been identified: COX-1 (constitutive form) and COX-2 (inducible form), which are the products of two different genes. The COX-1 enzyme is constitutively expressed and regulates normal physiological processes such as gastrointestinal, renal, and platelet function. COX-2, an inflammatory and inducible enzyme, is predominant with its expression seemingly androgen-dependent in rat distal vas deferens but not in testis, epididymis, prox vas, or prostate (McKanna *et al*., 1998). Standfield and Khan (Standfield and Khan., 2003) demonstrated that COX-2 was constitutively expressed in the initial segment of the epididymis, caput epididymidis and vas deferens at all stages of maturation. COX-2 can be induced by numerous growth factors and cytokines (Warner *et al*., 2004) and is in mediation of cellular growth regulation, prevention of apoptosis (Kujubu *et al*., 1991) and tumorigenesis (Lu *et al*., 1995). Recent studies have shown that COX-2 expression also mediates a variety of physiological responses within the organism; for example, tissue injury and/or inflammation (Tippetts *et al*., 1988).

The present results have demonstrated that, *in vitro*, arecoline causes reduction of sperm progressive motility and its parameters in a dose-dependent manner. It is estimated that the concentrations of arecoline used in the present *in vitro* study should be far above the average levels found in the betel quid chewers. Unfortunately, a reference on the average arecoline levels in blood of such betel quid chewers is not available so far, although it has been detected in human saliva at a level up to 140 μg/ml (Nair *et al*., 1985) and in neonatal cord serum at 0.004-1 μg/ml (Pichini *et al*., 2003). The much higher doses of arecoline were applied in order to evaluate its action on sperm motility that mimics the previous study of nicotine on sperm motility (Gandini *et al*., 1997). Therefore, it is not surprising that there is no significant reduction in sperm motility and its parameters when low doses of arecoline were tested. Since arecoline at a concentration less than 10 μg/ml did not elevate COX-2 expression in sperm cells, it might have no clinical relevance,

as shown in the present results that there was no statistical difference of sperm motility parameters between betel quid chewers and non-chewers. One of the reasons is probably that the process of spermatogenesis is too fast to be affected by such a low dose of arecoline. However, the most serious concern must be the inability to assess whether spermatogenesis in men is being affected adversely by betel quid before better and more sensitive tests or a massive epidemiological survey can be established. Recent technological developments have allowed for more reliable detection of arecoline in human saliva, but not blood or seminal fluid as yet, by high-performance liquid chromatography (Cox *et al*., 2004).

The present results demonstrate that, in human sperm cells, arecoline induces COX-2 expression in a dose-dependent fashion. It is, therefore, reasonable to suppose that this increased COX-2 protein might in turn be involved with the synthesis of the prostanoids, resulting in the subsequent pathological processes in the cells. A similar observation in human keratinocytes has shown that areca nut ingredients induce the production of PGs, COX-2 mRNA and protein formation (Jeng *et al*., 2000).

The effect of PGs on sperm motility is rather complicated. In seminal fluid, the average concentration of PGE was 44.3 times higher than that of $PGF2_{2\alpha}$. No relationship between $PGF_{2\alpha}$ and sperm motility was observed (Gottlieb *et al*., 1988; Cosentino *et al*., 1984) while $PGF_{1\alpha}$ reduced the sperm motility (Yang *et al*., 2001). On the contrary, a study of on 100 men demonstrated that $PGF_{2\alpha}$, in larger than physiologic levels, significantly reduced sperm motility (Cohen *et al*., 1977). It has been reported that there is no relationship between seminal PGE and sperm motility (Gottlieb *et al*., 1988; Bygdeman *et al*., 1970), although PGE might act to decrease calcium uptake into spermatozoa by elevating intercellular cAMP level

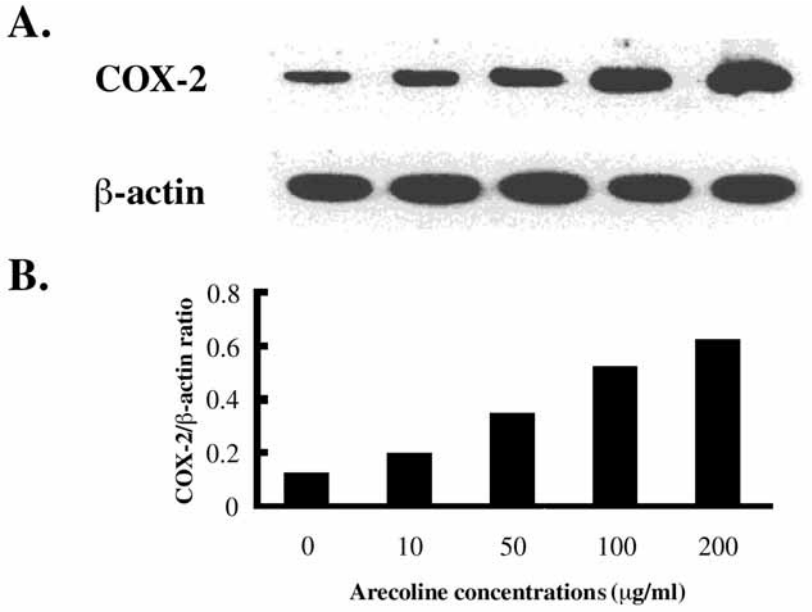

Fig. 2. (A), Expression of the COX-2 in human sperm cells after exposure to different concentrations of arecoline (10, 50, 100, 200 μg/ml) for 4 hours. 25 μg/ml of proteins from cell lysates were loaded onto a 10% SDS-PAGE, electrophorsed and subsequently transferred to nirocellulose membrane. The membrane was probed with monoclonal antibody specific for COX-2. β-actin showed as control integrity for proteins and loading. (B), The bands were examined with a computerized digital imaging system using AlphaImage 2200 software (Alpha Innotech, San Leandro CA). The integrated density value (IDV) was obtained as COX-2 protein to β-actin ratio by integrating all of the pixel values in the area of one band after correction of background.

(Peterson *et al*., 1980). In humans, there are significant elevations of PGE_2 and $PGF_{2\alpha}$ in the seminal plasma in diabetic men with significantly greater numbers of abnormal spermatozoa and significantly lower ability to penetrate hamster eggs compared with control subjects. Nevertheless, there is no difference in semen volume, sperm count, and spermatozoa motility between the two groups (Shrivastav *et al*., 1989). Prostaglandins (PGs) are also shown to influence sperm motility, contractility of the smooth muscle layers surrounding the seminiferous tubules and growth of both the seminal vesicle and the ventral prostate (Ellis *et al*., 1981). Indeed, this calls for a careful evaluation on the mechanisms underlying the abnormalities in spermatozoa, and the relationship between these abnormalities and increase in prostanoid concentrations. For example, PGE_2 at a concentration of 25 μg/ml is most effective in stimulating motility of washed human sperm, but does not work on fresh semen (Ellis *et al*., 1981). In addition, the prostaglandin inhibitor increased sperm count, sperm motility, and fertilizing capacity in male infertility (Colon *et al*., 1986). In contrast, Jessica HK *et al*. (Jessica *et al*., 2003) demonstrated that COX inhibitors decrease turkey sperm mobility. We suggest that arecoline is able to decrease sperm motility and its parameters because arecoline may interact with human sperm membrane and cause a significant decrease in sperm motility via the COX-2 pathway. On the other hand, effects of toxicity of arecoline or actions via other mechanisms cannot be ruled out.

In conclusion, one of the primary aims in this study is to examine the effect of betel quid chewing on male spermatogenesis based on the concept that the process of spermatogenesis is extremely vulnerable to adverse environmental effects. It appears that betel quid chewing does not affect spermatogenesis clinically. Nevertheless, we have shown *in vitro* that arecoline is able to reduce sperm motility and its parameters, as well as induce COX-2 expression in sperm cells. These facts simply bring a message to the millions of betel quid chewers that betel quid chewing might be harmful to the gonadal functions.

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