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# Murine sperm capacitation, oocyte penetration and decondensation following moderate alcohol intake

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## Abstract

**Male chronic alcohol abuse causes testicular failure and infertility. We analyzed the effects of moderate sub-chronic alcohol intake on sperm morphology, capacitation, fertilization and sperm head decondensation. CF-1 male mice were administered 15% ethanol in drinking water for 15 days; control mice received ethanol-free water. Similar patterns of tyrosine phosphorylation were observed in capacitated spermatozoa of control and treated males. Percentage of hyperactivation (H) and spontaneous (SAR) and progesterone-induced (IAR) acrosome reaction significantly decreased at 120 and 150 min of capacitation in treated males compared to controls (H: 14.1 ± 2.5 vs 23.7 ± 2.6, $P < 0.05$; SAR-T120 min: 17.9 ± 2.5 vs 32.9 ± 4.1, $P < 0.01$; IAR-150 min: 43.3 ± 3.5 vs 73.1 ± 1.1, $P < 0.001$, $n = 6$). During *in vitro* fertilization (2.5, 3.5 and 4.5 h post-insemination), there was an increased percentage of fertilized oocytes (with a decondensed sperm head and one or two pronuclei) in treated males ($P < 0.001$, $n = 7$). After 60 min of *in vitro* decondensation with glutathione plus heparin, the percentage of decondensed sperm heads was significantly higher in treated males than in controls (mean ± S.D.: 57.1 ± 5.6 vs 48.3 ± 4.5, $P < 0.05$, $n = 5$). The percentage of morphologically normal sperm heads was significantly decreased in treated males with respect to controls ($P < 0.001$, $n = 9$). These results show that short-term moderate alcohol consumption in outbred mice affect sperm morphology, hyperactivation, acrosomal exocytosis, and the dynamics of *in vitro* fertilization and *in vitro* sperm nuclear decondensation.**

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## Introduction

It is widely known that alcohol abuse produces a variety of medical, psycho-sociological and physiological disorders at different levels, including the reproductive system (Canteros *et al*. 1995, Cebral *et al*. 1997, 1998*a*,*b*, 2011, Lee *et al*. 2010). Numerous studies have shown the deleterious effects of chronic heavy alcohol consumption on testicular function and spermatogenesis (Shayakhmetova *et al*. 2013, 2014), on testosterone serum levels (Salonen *et al*. 1992, Muthusami & Chinnaswamy 2005, Lee *et al*. 2010, Jensen *et al*. 2014, Sliwowska *et al*. 2016) and on hypothalamic-pituitary-testicular axis function (Cicero & Badger 1977, Dees *et al*. 1990, Välimäki *et al*. 1990, Salonen *et al*. 1992, Zhang *et al*. 2005). Clinical manifestations in alcoholic men include hypogonadism, testicular atrophy, feminization, sexual dysfunction (Van Thiel *et al*. 1990, Van Heertum & Rossi 2017), infertility and delayed sexual maturation (Anderson *et al*. 1989). Many studies have suggested that one of the most important negative effects of chronic alcohol ingestion occur in sperm parameters (Rahimipour *et al*. 2013,Condorelli *et al*. 2015).

Reproductive function in the ethanol-disrupted male depends on alcohol concentration and duration of ethanol exposure, issues that remain controversial. A decrease in the quality of semen parameters has been consistently documented in heavy consumers of alcohol (Martini *et al*. 2004, Muthusami & Chinnaswamy 2005, Gaur *et al*. 2010, Hansen *et al*. 2012). On the other hand, the effects of low to moderate consumption of alcohol do not appear to be clinically significant. Several studies have shown no effect in semen parameters with moderate alcohol consumption (Stutz *et al*. 2004, Van Heertum & Rossi 2017). de Jong *et al*. (2014) failed to show a significant relationship between alcohol consumption and not only sperm parameters but also pregnancy outcome. Others have observed a


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positive effect of male alcohol intake on fertilization rate (Firns *et al*. 2015). Regarding duration of alcohol exposure, multiple studies have showed altered sperm morphology with regular alcohol drinking, correlating with low fecundity (Van Heertum & Rossi 2017). Chronic consumption of 5% ethanol for 20 weeks or 6% ethanol for 5 weeks (that produced 45% ethanol-derived calories (EDC)) led to low caudal sperm content in rats (Anderson *et al*. 1983, Willis *et al*. 1983). Consumption of 5% ethanol for 6 weeks induced increased percentage of morphologically abnormal spermatozoa (Abel & Moore 1987). Most of the studies that have analyzed the effects of alcohol intake on testicular and sperm parameters have used chronic alcohol intake paradigms and alterations in murine male fertilizing ability following short-term moderate ethanol consumption remain unclear. We have previously shown that chronic ingestion of 5% alcohol (in drinking water) for 30 days by hybrid (C57/Bl × CBA) F1 adult male mice did not affect *in vitro* fertilization (IVF; Cebral *et al*. 1997). Considering that the CF-1 outbred mouse has a high genetic polymorphism and variability in terms of biological responses, this mouse colony could provide a feasible model to identify effects on sperm function and fertility that resemble those observed in alcoholic men who consume alcohol in a sub-chronic and moderate manner.

The cellular mechanisms underlying the morphological and physiological changes in spermatozoa and fertility following male alcohol ingestion have been little studied. Alcohol consumption may affect, directly or indirectly, spermatogenesis, differentiation/elongation of spermatids, compaction of sperm chromatin and sperm epididymal maturation, and thus, negatively alter capacitation, hyperactivation and acrosome reaction and ulterior events of fertilization. Both acute and chronic alcohol consumption have been shown to affect sperm chromatin/DNA integrity and apoptosis (Talebi *et al*. 2011, Rahimipour *et al*. 2013), and result in the production of sperm with a less compacted chromatin, suggesting that these alterations could be one of the possible causes of infertility due to alcohol consumption.

Considering the lack of information regarding the effect of sub-chronic alcohol ingestion on the sperm fertility, the aim of this paper was to analyze its effect on sperm capacitation and associated functional parameters, on the dynamics of oocyte penetration and sperm head decondensation, and on its potential relationship with altered sperm morphology, in an outbred adult mouse model. Our hypothesis is that moderate alcohol intake for a short period of time negatively affects sperm hyperactivation and acrosome exocytosis and that the kinetics of *in vitro* oocyte fertilization is altered by changes in time course of sperm head decondensation.

## Materials and methods

### *Animals*

Outbred CF-1 sexually mature mice (*Mus musculus,* CrlFcen:CF1, Mouse Genome Informatic (MGI)), produced by FCEN (School of Exact and Natural Sciences) of the University of Buenos Aires (Buenos Aires, Argentina) were housed by sex in groups of three to four mice per cage. They were kept in controlled room temperature (22°C) and light cycle (14 h light/10 h dark) and were fed commercial mouse chow (*Alimento* '*Balanceado Cooperación Rata-Ratón*' from the *Asociación Cooperativa de Alimentos S.A. Buenos Aires, Argentina*) and tap water *ad libitum*. At the outset of ethanol treatment, CF-1 male and female mice were 60 days old, with average body weight between 27 and 30 g.

### *Ethanol treatment and assessment of ethanol intake and blood alcohol concentration*

These experiments were carried out in accordance to regulations and ethical standards of Institutional Animal Care and Use Committee (IACUC, protocol Nr 57), from Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires (FCEN-UBA Argentina), and in accordance with the guidelines of the National Institutes of Health (NIH).

Adult male mice were given access to 15% (v/v) ethanol in drinking water for 15 days (treated males) *ad libitum*. In a previous study, conditions for short-term moderate alcohol ingestion in the male were established by determining the ethanol concentration producing an adverse effect on morphology and functionality of epididymal spermatozoa. 5, 10 or 15% ethanol was administered in drinking water for 15 days to CF-1 adult mouse. The concentration showing deleterious effects on sperm morphology and testes was 15% (data not shown). Control males received ethanol-free drinking water *ad libitum*. To monitor potential ethanol-related nutritional effects on body weight of treated mice, a group of animals were pair-fed for 15 days with standard commercial food and the same daily percentage of ethanol-derived calories (% EDC) in drinking water as in treated animals. Water-control and pair-fed control mice consumed the same amount of liquid and food during the 15 days of the experiment, and no changes in body weight were detected between both groups at the end of treatment. Thus, water-control was chosen as the control group for subsequent experiments.

Control and treated males were weighed at the beginning and at the end of ethanol treatment. Every morning, the drinking volume and the quantity of food consumed were recorded to monitor the amounts of daily liquid, food, calorie intake (estimated by caloric value of the diet used (3976 kcal/kg) and EDC, estimated as 7.1 kcal/g). From these data, the mean calorie intake and the percentage of EDC were estimated for each experimental group. At least 5 mice per experimental group were used in each experiment. On the morning of day 15 of ethanol administration, CF-1 males were killed by cervical dislocation and trunk blood was collected into heparinized Eppendorf tubes. Blood samples were held at 4°C for ethanol measurement using a commercial kit

(NAD/NADH enzymatic assay, sensitivity 50.1 mg/dL), within 4 h of collection. Blood alcohol concentration (BAC) was expressed as milligrams per deciliter.

### *Epididymal sperm preparation*

Male mice were killed by cervical dislocation on the morning of day 15 of ethanol treatment. The epididymides of each male were dissected and both *caudae* were removed and transferred into a dish containing 400 µL of *In Vitro* Fertilization Medium (IVFM: 99.3 mmol/L NaCl, 2.70 mmol/L KCl, 0.50 mmol/L $MgSO_4$ .$6H_2O$, 1 mg/mL glucose, 0.31 mmol/L $Na_2HPO_4$.$2H_2O$, 1.80 mmol/L $CaCl_2$.$H_2O$) (Fraser & Drury 1975), without bovine serum albumin (BSA). pH of the medium was adjusted to 7.3 with 25.07 mmol/L NaOH, and 0.0055 mg sodium pyruvate and 0.35 mL L-Na-lactate (60% syrup) were added to a final volume of 100 mL. Spermatozoa were obtained by making small incisions in *caudae* tissue and allowing the dense mass of spermatozoa to disperse for 5 min at 37°C. Tissue fragments were removed; the sperm suspension was homogenized, an aliquot of spermatozoa was collected to analyze sperm count and morphology, and spermatozoa were then incubated in capacitating conditions (supplemented with 3 mg/mL of BSA, Sigma Chemical), for 120 min at a final concentration of $2 \times 10^6$ spermatozoa/mL, according to Visconti *et al.* (1995). Another aliquot ($2 \times 10^6$ spermatozoa) was kept in non-capacitating conditions (without BSA).

### *Protein extraction and immunodetection of phosphotyrosine residues after capacitation*

After capacitation, sperm suspensions from 5 control and 5 treated males were centrifuged at 2800 ***g*** for 1 min. Supernatants were discarded and pellets washed once with 1 mL PBS, for 2 min at 5000 rpm at 37°C. Supernatants were discarded leaving the cells in about 20 µL. Following addition of enough 6× Laemmli's loading buffer, containing 5% β-mercaptoethanol and Bromophenol blue, samples were heated at 100°C, for 5 min and centrifuged at 11,200 ***g*** for 5 min. Supernatants were kept at −20°C until further processed.

Sperm extracts, equivalent to $2 \times 10^6$ cells, were analyzed in 10% SDS-PAGE, with a 4% stacking gel, according to Laemmli (1970), at a constant current of 25 mA per gel, at room temperature. Molecular weight markers were loaded in one of the lanes.

Following electrophoresis, proteins were transferred onto a PVDF membrane, according to the method of Towbin *et al.* (1979), at a constant voltage of 110V, for 1 h at 4°C. Transfer efficiency was checked by Ponceau Red staining. Phosphotyrosine containing bands were detected using an anti-phosphotyrosine antibody (Upstate Biotechnology Incorporated, NY, EEUU, Cat # 05-321). Following incubation in blocking solution (0.1% BSA, 0.4% Tween-20, 1 mM EDTA in TBS), for 1 h, the membrane was incubated in monoclonal anti-phosphotyrosine antibody at a 1:1000 dilution in blocking solution, for 1 h, at room temperature. After 4 washes, 5 min each, with PBS-0.1% Tween-20, the membrane was incubated with second antibody (peroxidase-labeled rabbit mouse anti IgG, in a 1:5000 dilution), for 1 h, at room temperature, in blocking solution. The membrane was finally washed as previously described and reactive bands were detected using an ECL kit (Amersham Life Science).

### *Sperm morphology determination*

To evaluate sperm head and flagellum morphology, 10 µL of caudal sperm suspension was placed on a slide. Immediately after drying, slides were fixed for 15 min in 5% formaldehyde in phosphate buffer (v/v), washed in distilled water and stained with the *Spermac* staining procedure (Spermac Stain Enterprises, Onderstepoort, South Africa), according to the manufacturer's instructions. Slides were examined at ×100 under an Axiophot Zeiss microscope (Carl Zeiss) equipped with a camera driven by Olympus DP71 using an image analyzer Olympus cell Sens software (Olympus). Head morphology, acrosomal and post-acrosomal regions, cytoplasmic droplet, midpiece and flagellum were examined. The criteria for abnormal sperm morphology used were as follows: (1) abnormal head: increased or decreased size, flat head, partial or completely abnormal shape (round, small, large, double head), and/or abnormal acrosome (acrosome more than 30% smaller or 70% larger than sperm head); (2) neck and midpiece defects: debris around the neck, thickened neck, midpiece measuring more than 30% of spermatozoa; (3) abnormal flagellum: double, coiled or broken flagellum, incorrect insertion of flagellum, presence of a cytoplasmic droplet. The mean percentage ± S.D. (over a total 1000 spermatozoa evaluated/mouse) of abnormal sperm heads, neck-midpieces and flagella was calculated for 9 control and 9 treated males (Cebral *et al.* 2011).

### *Sperm motility and hyperactivation assessment*

Sperm motility and hyperactivation were evaluated at 0, 60 and 120 min of *in vitro* capacitation. Immotile, motile and hyperactivated spermatozoa were determined using a *Neubauer* chamber. Motility was expressed as the mean percentage of motile spermatozoa/total number of spermatozoa, and hyperactivation was expressed as the mean percentage of hyperactivated spermatozoa over the total number of motile spermatozoa ± standard error of the mean (S.E.M.). 6 males were evaluated in each group.

### *Acrosomal reaction analysis*

The *HOS-Spermac* assay allows simultaneous detection of acrosome-reacted spermatozoa and motile spermatozoa. Briefly, different aliquots of sperm suspension were collected every 60 min from time zero ($t = 0$) up to 120 min of capacitation (time course of spontaneous acrosomal reaction). At 120 min, 15 µM progesterone (final concentration) (Sigma Chemical Company) was added to the sperm suspension and incubated for an additional 30 min to evaluate induced acrosomal exocytosis at 150 min. At each time point, acrosomal status (presence (blue band stained in sperm head) or absence of acrosome (pink head)) was evaluated by the *HOS-Spermac* method in motile spermatozoa (Herrero *et al.* 1998). Briefly, 20 µL of the sperm suspensions was transferred to Eppendorf tubes containing 200 µL hypoosmotic medium (HOSM = 0.735 g

sodium citrate and 1.351 g fructose in 100 mL distilled water) and incubated for one hour at 37°C. Then, spermatozoa were pelleted by centrifugation at 500 ***g*** for 5 min and resuspended in 20 µL HOSM. Ten microliters of this suspension were placed on a microscope slide and allowed to dry. Immediately after drying, slides were fixed for one hour in 5% formaldehyde in phosphate buffer (v/v), washed with distilled water, stained with the *Spermac* procedure and allowed to dry. Slides were examined in a bright field microscope at ×100, under immersion. Viability and the presence of the acrosomal cap were assessed in at least 200 spermatozoa per control and treated males (6 mice per group). Results were expressed as mean percentage reacted spermatozoa ± S.E.M.

### In vitro *decondensation*

After 120 min of capacitation, *in vitro* sperm nuclear decondensation was induced in the presence of 100 µmol/L glutathione (GSH) plus 4.6 µmol/L heparin (Hep, 13,500 kDa, 170 IU/mg) for 30 and 60 min. Controls consisted of parallel incubations with GSH or heparin alone. After each time period, a 30-µL aliquot of the sperm suspension was removed and fixed with an equal volume of 2.5% glutaraldehyde in phosphate-buffered saline (PBS). Aliquots of 5 µL spermatozoa were stained (Hoechst *33342*, 0.5 µg/mL, Sigma) or not, and transferred onto a slide to assess nuclear status under phase contrast and fluorescence microscopy (Olympus CH2 microscope at 40×). Spermatozoa were classified as unchanged (U), moderately decondensed (M) or grossly decondensed (G) (Sanchez *et al.* 2013). At least 200 cells were evaluated in each category. Total sperm decondensation achieved was determined as the total % M plus % G (% M+G) and data were expressed as mean % values ± standard deviation of the mean (S.D.M.) for 5 males per group.

### In vitro *fertilization (IVF)*

Male mice were killed by cervical dislocation on the morning of day 15 of ethanol treatment, to perform IVF-experiments (Cebral *et al.* 1997). The epididymides of one male were dissected and the *caudae* placed into 300 µL drop of IVFM-3% BSA, overlaid with mineral oil to collect the sperm suspension, as described earlier. Spermatozoa were then incubated for 90 min at 37°C and 5% $CO_2$ in air, to allow for capacitation.

Adult female mice were induced to superovulate with 5 IU of pregnant mare's serum gonadotrophin (PMSG, Sigma) given at 18:00 h on day 12 of ethanol treatment, and 5 IU of human chorionic gonadotrophin (hCG, Sigma) 48 h later. Females were killed by cervical dislocation 16–17 h after hCG injection when ethanol treatment was stopped (day 15 of treatment). Both oviducts were removed and placed in PBS. One oocyte-cumulus complex (OCC) was released from each ampulla into a 50-µL drop of IVFM-BSA (one cumulus mass per drop) and overlaid with mineral oil.

One OCC from one female was inseminated with $1–2 \times 10^5$ spermatozoa/mL from a control male, and the second OCC of the same female was inseminated with the same concentration of spermatozoa from a treated male. With this experimental design, 7 IVF-experiments were performed, with a total of 3 females and one control and one ethanol-treated male per experiment, and with a final number of 21 females and 7 control and 7 treated males.

The timing of IVF events was evaluated in control and treated males after examination of oocytes at 2.5, 3.5 and 4.5 h of *in vitro* insemination. Oocytes were washed to remove cumulus cells and adherent spermatozoa and were then fixed with 2% paraformaldehyde and incubated for 10 min with *Hoechst 33342* (0.5 µg/mL) in PBS. After washing (PBS), oocytes were examined under phase contrast and fluorescence microscopy (Nikon ActurusXT microdissection microscope) and classified as follows (Fig. 1): (a) fertilized oocytes: Telophase II (Te II) oocytes with second polar body (2PB's) and a decondensed head in the ooplasm (DH); oocytes with 2PB's + female pronucleus (fPN) + male pronucleus (mPN); (b) unfertilized oocytes: metaphase II-arrested oocytes (Me II); Me II-arrested oocytes with adhered spermatozoa (As); oocytes with 2PB's and fPN (spontaneously activated oocytes with second polar body and one female pronucleus); fragmented oocytes.

The number of fertilized and unfertilized oocytes, the number of oocytes with a decondensed sperm head and the number of oocytes with 2PB's and 2PN's were recorded for each control and treated male at each time point studied. Results were expressed as the mean % oocytes in each category ± S.D.M.

### *Statistical analyses*

Reported values of control and treated derived samples were expressed as mean ± standard deviation of the mean (S.D.M.) or standard error of the mean (S.E.M.), as stated in text. Differences between group means were statistically analyzed by one-way analysis of variance (ANOVA) and Student's *t* test. Differences between groups were considered statistically significant when $P < 0.05$. Analyses were performed with GraphPad InStat v2.05a (GraphPAD Software).

## Results

### *Male mouse body weight, food and liquid consumption and blood alcohol concentration after sub-chronic ethanol treatment*

Given that changes in body weight and patterns of liquid and food intake have not been previously monitored in a model of male sub-chronic 15% alcohol intake, and since ethanol consumption could alter these parameters, they were monitored daily for the whole duration of the treatment. Treated males consumed significantly lower quantities of food than controls ($P < 0.05$, Table 1), but the total calorie intake was similar in both groups. Treated males consumed 30 g/kg/day ethanol and had 30.3% EDC (Table 1). No differences in body weight between treated males and controls were registered either at the beginning or at the end of ethanol treatment. The level of BAC in treated males was 15–60 mg/dL, and no alcohol was detected in control males.

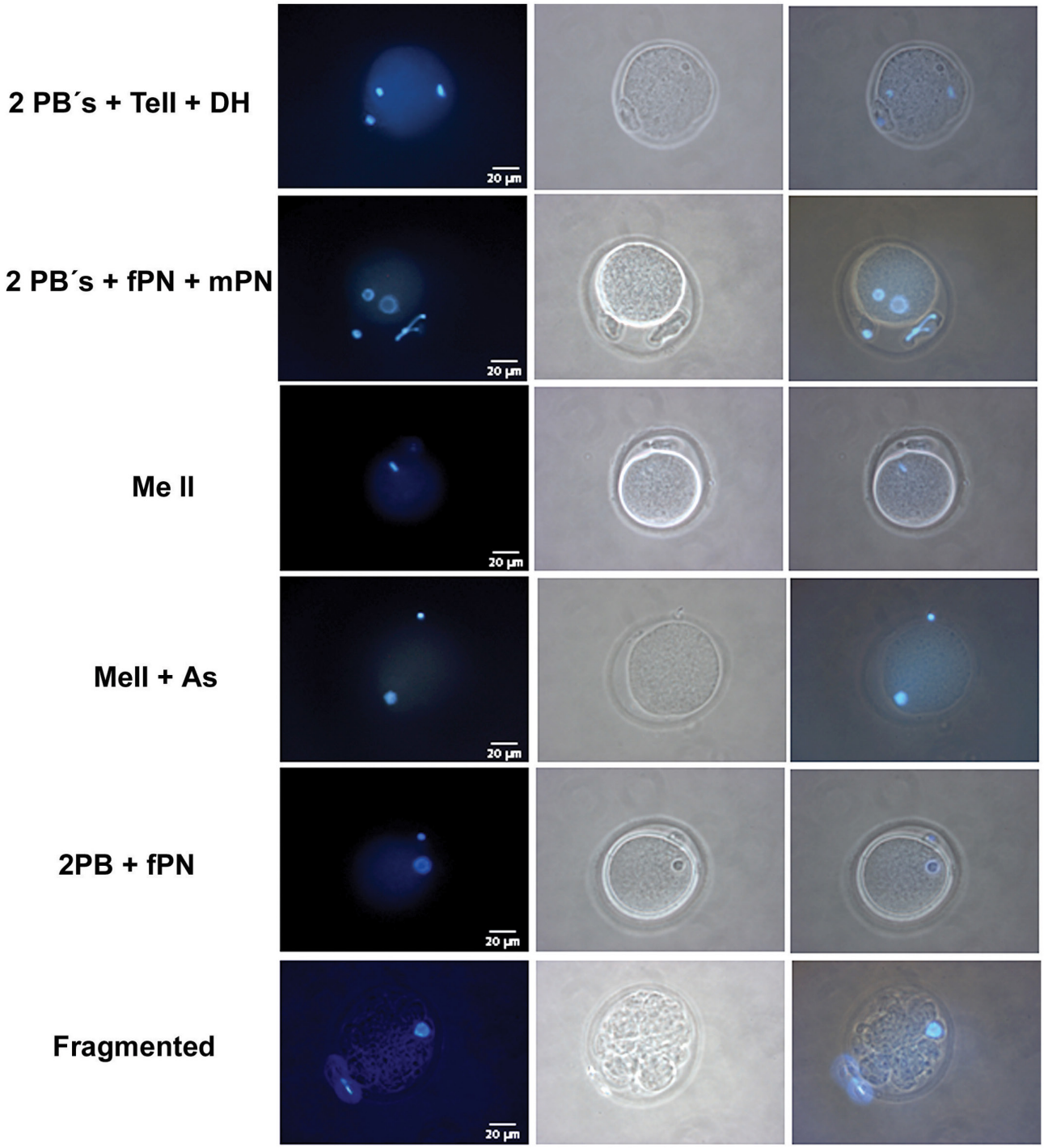


**Figure 1** CF-1 murine oocyte evaluation after *in vitro* fertilization. After *in vitro* fertilization (IVF), oocytes were examined under phase contrast and fluorescence microscope and classified as: telophase oocytes (Te II) with second polar body and a decondensed sperm head in the ooplasm (2PB's + Te II + DH); oocytes with female and male pronucleus (2PB's + fPN + mPN); metaphase II-arrested oocytes (Me II); Me II-arrested oocytes with adhered spermatozoa (Me II + As); spontaneously activated oocytes (2PB's + fPN) and fragmented oocytes. First column: nuclear *Hoechst* fluorescence; second column: phase contrast; third column: merge.

**Table 1** Alcohol intake pattern and body weight in control and treated males.

| | Control males ($n=15$) | Treated males ($n=15$) |
|---|---|---|
| Food intake (g/kg/day) | 200.0 ± 16.9 | 163.0 ± 11.2* |
| Liquid intake (mL/kg/day) | 222.5 ± 33.8 | 200.1 ± 19.7 |
| Food calories (kcal/kg/day) | 610.1 ± 50.7 | 479.0 ± 33.6* |
| Liquid calories (kcal/kg/day) | – | 213.1 ± 20.9 |
| Total calories (kcal/kg/day) | 610.1 ± 51.7 | 692.0 ± 54.6 |
| Ethanol intake (g/kg/day) | – | 30 g/kg/day |
| % EDC | | 30.3% |
| Body weight | | |
| Initial weight (g) | 34.4 ± 0.56 | 34.1 ± 0.8 |
| Final weight (g) | 35.2 ± 0.5 | 34.0 ± 1.0 |

Mean daily food and liquid intake (g, mL/kg/day) and body weight (g) were assessed at the beginning, during and at the end of sub-chronic 15% ethanol administration, in both, control (no ethanol administration) and treated males. Energy intake was calculated from food and ethanol consumption (kcal/kg/day). Mean ethanol intake is expressed in g/kg/day and % EDC (percentage of ethanol-derived calories). Values are expressed as mean ± S.E.M.
*$P<0.05$ vs control males, Student's *t* test, 15 animals per group.

### *Capacitation, hyperactivation and acrosomal exocytosis after sub-chronic alcohol consumption*

Up to date, there is no evidence on the effects of sub-chronic moderate alcohol consumption on sperm capacitation and associated parameters in the adult mouse model. Initially, we analyzed protein phosphorylation on tyrosine residues, considered by several authors as a possible indicator of sperm capacitation (Jabbari *et al*. 2009). Accordingly, we analyzed the pattern of tyrosine phosphorylation in spermatozoa from alcohol-treated mice and controls, following incubation in capacitating conditions. Figure 2A shows a Western blot analysis of the protein pattern for phosphotyrosine expression, after 120 min of incubation under capacitating conditions. A different pattern of phosphorylated bands could be observed between non-capacitated and capacitated spermatozoa from both control and treated groups. However, the same bands of MW p95/116 (hexokinase), 72/73, 50/51, 26/27 and 20/21 kDa, were observed under capacitating conditions in both control and treated animals, indicating that alcohol consumption did not affect the expression of phosphorylated proteins after capacitation.

Although we did not find noticeable differences in the pattern of protein tyrosine phosphorylation between control and treated animals, we decided to study hyperactivation and acrosome reaction, both capacitation-related phenomena, since other factors could be involved in their regulation. We first analyzed sperm motility following incubation *in vitro* in capacitating conditions. In treated males, the percentage of motile spermatozoa was not significantly different than that of control animals (Fig. 2B). However, hyperactivation was significantly reduced in spermatozoa from treated males as compared to controls, at 60 and 120 min of capacitation (T60 min: $P<0.05$, T120 min: $P<0.05$, Fig. 2C).

Subsequently, we analyzed acrosomal exocytosis in motile spermatozoa in both groups. The percentage of spermatozoa that underwent spontaneous loss of acrosomal content was significantly reduced in treated males at 120 and 150 min of capacitation as compared to control males (T120 min: $P<0.01$; T150 min: $P<0.001$, Fig. 2D). In addition, while control-derived spermatozoa showed an increase ($P<0.05$) in acrosomal exocytosis after exposure to 15 µM progesterone at 150 min of capacitation, the percentage acrosome reaction in spermatozoa from treated males was significantly reduced in comparison to control value ($P<0.001$, Fig. 2D).

### In vitro *oocyte penetration and sperm head decondensation kinetics after sub-chronic alcohol consumption*

IVF studies allow for a detailed analysis of sperm penetration and kinetics of head decondensation at early post-insemination times. The frequencies of fertilized and unfertilized oocytes at 2.5, 3.5 and 4.5 h post-*in vitro* insemination were studied following 15 days of 15% alcohol administration to CF-1 male mice. The mean percentage of fertilized oocytes (with 2PB's and female PN plus decondensed head or with 2PNs, Fig. 1) significantly increased from 2.5 to 3.5 h but not from 3.5 to 4.5 h after *in vitro* insemination, both in control and treated males. However, at each IVF time point evaluated, the percentage of fertilized oocytes was significantly higher in treated vs control males ($P<0.001$, Fig. 3A). Consequently, at each time point, the percentage of unfertilized oocytes was significantly reduced in treated vs control males ($P<0.001$, Fig. 3B).

To elucidate whether the increased percentage of fertilized oocytes of treated males was due to a change in the time-pattern of sperm decondensation, we analyzed sperm decondensation kinetics after IVF in both control and treated groups. The percentage of fertilized oocytes with a decondensed head (Fig. 1) significantly increased in treated males at 2.5 h of *in vitro* insemination ($P<0.001$), but reduced at 3.5 h, when compared to control animals ($P<0.001$, Fig. 4A). However, at 3.5 and 4.5 h after insemination, the mean percentage of fertilized oocytes with 2PB's and 2PN's (Fig. 1) in treated males was significantly increased compared to control males ($P<0.001$, Fig. 4B).

We have previously shown (Sanchez *et al*. 2013) that mouse sperm decondensation can be achieved, *in vitro*, using glutathione (GSH) and heparin and, thus, we further analyzed *in vitro* decondensation kinetics in both control and treated males. Figure 5 shows the percentage of decondensed spermatozoa from control and treated males in the presence of GSH and/or 4.6 µM heparin. As expected, the addition of GSH or heparin alone did

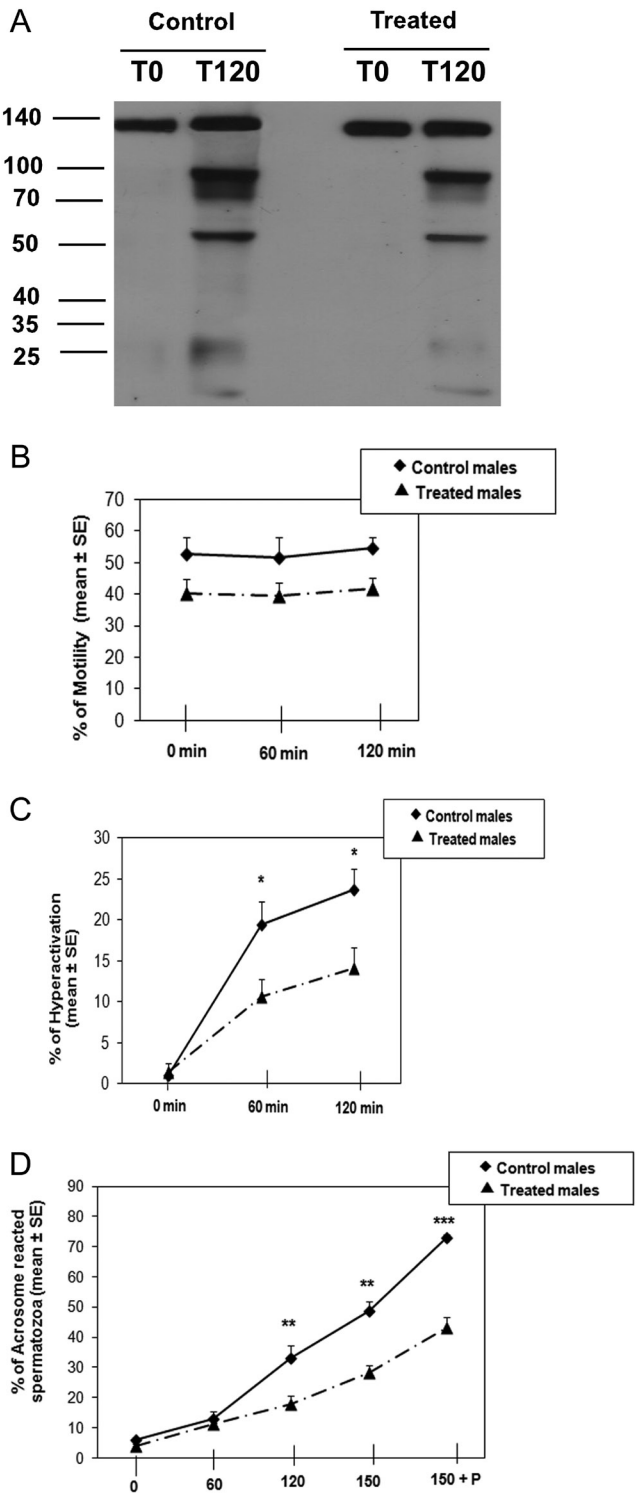


**Figure 2** Sperm capacitation following sub-chronic alcohol consumption in CF-1 mice. (A) Analysis of tyrosine protein phosphorylation in spermatozoa from control and treated males. Protein tyrosine phosphorylation of caudal epididymal mouse sperm was evaluated after incubating $2 \times 10^6$ spermatozoa/mL in capacitating conditions for 120 min. Proteins were extracted and separated by 10% SDS-PAGE and identified using a specific anti-phosphotyrosine antibody. Both control and treated males showed a similar pattern of protein phosphorylation when sperm cells were incubated under capacitating conditions for 120 min. Left lane: molecular weight markers. T0: non-capacitated spermatozoa. A representative Western blot membrane is shown. The experiment was performed 5 times, with similar results. A total of 5 animals per group were used. (B and C) Motility and hyperactivation during capacitation in control and treated males. Murine spermatozoa were recovered from epididymal caudae of control (continuous line) and treated males (dotted line) and sperm motility % (B) and hyperactivation % (C) were determined. Values are expressed as mean ± standard error of the mean (S.E.M.). Percentages of hyperactivated spermatozoa were significantly diminished in treated males vs controls at 60 and 120 min of capacitation (*$P<0.05$, 6 males per group, Student's *t* test). (D) Spontaneous and induced acrosomal exocytosis in control and treated males. The presence or absence of acrosomal vesicle was evaluated at 0, 60, 120 and 150 min of capacitation by the *HOS-SPERMAC* procedure, as indicated in Materials and methods. At 120 min, progesterone (15 µM final concentration) was added to sperm incubation to induce acrosome reaction (150 + P). Acrosome reaction % was calculated as number of acrosome-reacted sperm over total number of vital spermatozoa (hypoosmotic test). Results are expressed as mean ± S.E.M. Spermatozoa from treated males (dotted line) presented a significant decrease in spontaneous acrosome reaction at 120 and 150 min of capacitation (**$P<0.01$) and progesterone-induced acrosome reaction at 150 min (***$P<0.001$) as compared to control values (continuous line) (6 males per group, Student's *t* test).

not produce decondensation, but GSH plus heparin induced sperm decondensation in both control and treated males. At 60 min of incubation, the percentage of decondensed spermatozoa from treated males was significantly increased as compared to control animals ($P<0.05$, Fig. 5).

### *Effects of sub-chronic alcohol consumption on sperm morphology*

Given that the changes in sperm functional parameters, oocyte penetration and sperm decondensation during fertilization could result from morphological alterations of the sperm head, we evaluated changes in acrosome morphology, head and midpiece shape and assessed the frequency of abnormal spermatozoa after sub-chronic ethanol intake. In contrast to morphologically normal sperm heads (Fig. 6A), an abnormal neck insertion (Fig. 6B), a sperm head with abnormal (smaller or absent) acrosome (Fig. 6C), a decreased head size and altered head shape (round; Fig. 6C) and a spermatozoon with cytoplasmic droplet and abnormal head shape (Fig. 6D) were recorded as morphological abnormal. Treated males had a significantly higher percentage of abnormal sperm head morphology as compared to controls ($P<0.001$, Table 2).

## Discussion

The aim of the present study was to analyze the effects of short-term moderate alcohol consumption on sperm

capacitation and IVF events in the outbred adult mouse, with emphasis on sperm head decondensation.

The effects of alcohol consumption in humans are not easy to assess because of the difficulties in comparison of populations, mainly due to variations in the pattern of alcohol intake. Because of intrinsic differences between humans and mice, no single mouse model can represent all features of a complex human trait such as alcoholism. Our results in an outbred mouse model demonstrated that short-term moderate ethanol consumption leads to altered sperm fertility, in accordance with other ethanol mouse models (Anderson *et al*. 1983, Morton *et al*. 2014, Wieczorek *et al*. 2015). One important feature of the present ethanol intake model in drinking water is that CF-1 mice reliably drank ethanol to moderate BAC levels (range 15–60 mg/dL) and consumed 30 g ethanol/kg body weight daily, similar to other paradigms of ethanol drinking intake with 10, 20 or 30% ethanol that produce an average BAC of approximately 1.6 mg/mL (Rhodes *et al*. 2005). With this value of moderate BAC levels, we found changes in fertilization outcome even though other authors did not find effects on reproductive parameters (Ogilvie *et al*. 1997, Bonthius *et al*. 2002, Zhang & Chong 2016). Furthermore, although the value of 30.3% EDC obtained was relatively low compared to value ranges previously reported (Willis *et al*. 1983, Abel & Moore 1987, Shirai & Ikemoto 1992, Mittleman *et al*. 2003), we observed not only an effect on fertilization but also altered sperm morphology and capacitation parameters.

In addition to ethanol concentration and routes of administration, ethanol effects on male reproductive function depend on the duration of exposure. In this work, we evaluated whether a moderate 15% ethanol concentration in the drinking water administered for 15 days had a deleterious effect on sperm morphology,

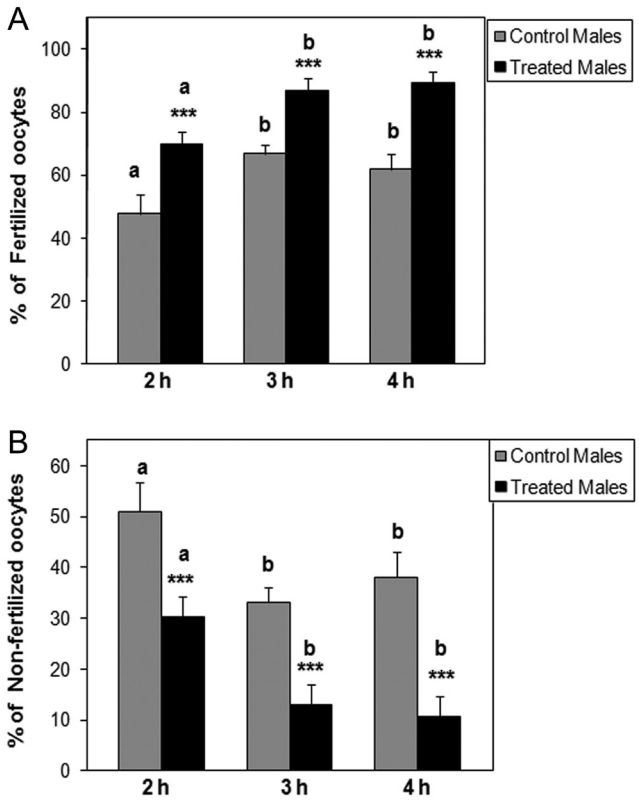


**Figure 3** Frequency of fertilized and unfertilized oocytes after IVF in control and treated males. (A) Fertilized oocytes: oocytes with 2PB's, female PN and a decondensed sperm head. At 2.5, 3.5 and 4.5 h after insemination, percentage of fertilized oocytes in the alcohol-treated group was significantly increased with respect to control (***$P<0.001$, Student's $t$ test). (B) Unfertilized oocytes: metaphase II-arrested oocytes, activated oocytes with 2PB's and a female pronucleus or with adhered spermatozoa. Percentage of unfertilized oocytes in the alcohol-treated group was significantly reduced with respect to control (***$P<0.001$, Student's $t$ test). Different letters above group bars denote significant differences between groups (A vs B: $P<0.01$, ANOVA, Student–Newman–Keuls test; 7 males per group). Results are expressed as mean ± standard deviation of the mean (S.D.M.).

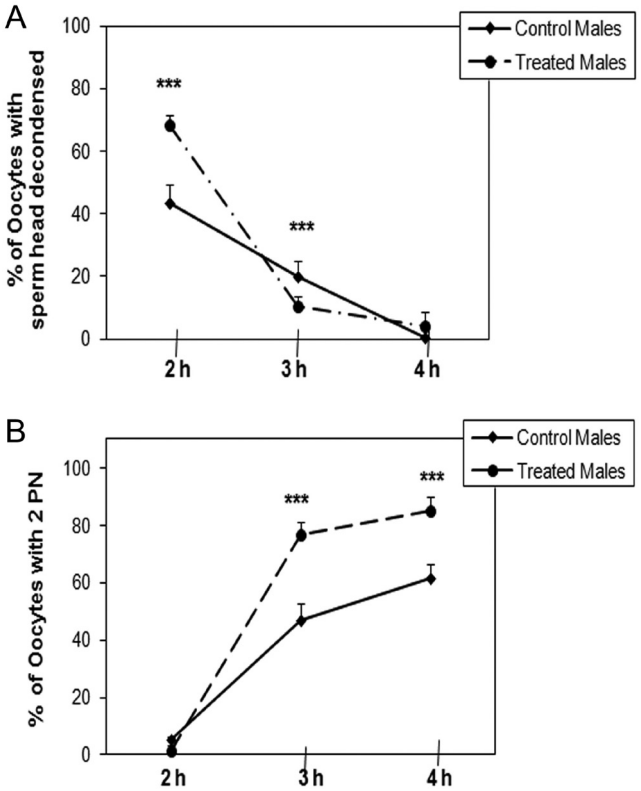


**Figure 4** Sperm head decondensation and pronuclear formation during *in vitro* fertilization in control and treated males. At 2.5, 3.5 and 4.5 h after insemination, fertilized oocytes stained with *Hoechst 33342* were analyzed for nuclear evaluation, as described in Materials and methods. (A) Kinetics of sperm head decondensation in each group was determined by calculating the percentage of oocytes with 2PB's, female pronucleus and a decondensed sperm head. (B) Kinetics of pronuclear formation in each group was determined by calculating the percentage of oocytes with 2PB's and 2PN's. ***$P<0.001$ vs control males, Student's $t$ test, $n=7$ males. Results are expressed as mean ± S.D.M.

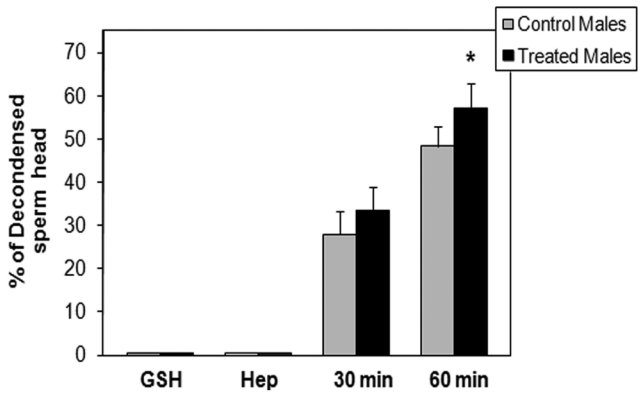


**Figure 5** Sperm head decondensation in control and treated males. *In vitro* nuclear decondensation was analyzed following incubation of spermatozoa from each group in glutathione (GSH) plus heparin 4.6 µM (Hep) at 120 min of capacitation and visualized by *Hoechst* staining. Negative controls: spermatozoa incubated with GSH or heparin alone. The percentage of moderately decondensed and grossly decondensed spermatozoa was analyzed at 30 and 60 min of incubation. At 60 min, sperm head decondensation in treated males was significantly higher than in controls (*$P < 0.05$, $n = 8$ males, Student's *t* test). Results are expressed as mean ± S.D.M.

sperm functional parameters and IVF dynamics. Considering that during mouse spermatogenesis (35 days duration) (Creasy & Chapin 2014), the spermatid elongation phase is about 8.5 days long , and that it takes an additional 4–5 days for sperm to reach the caudal epididymis (De Grava Kempinas & Klinefelter 2014), epididymal caudal spermatozoa were exposed to ethanol intake during the spermatid elongation phase in the seminiferous epithelium for about 10 days and during sperm maturation for an additional 5 days of epididymal transit, accounting for a 15-day ethanol exposure. Since chromatin head condensation takes place both during spermatid elongation in the testis and epididymal sperm maturation (Fujii & Imai 2014), the present short period of ethanol exposure could affect sperm head condensation.

The mechanisms involved in ethanol-induced infertility regarding alterations in sperm motility, capacitation or nuclear decondensation during IVF remain poorly understood (Pajarinen *et al*. 1996, Auger *et al*. 2001, Martini *et al*. 2004, Muthusami & Chinnaswamy 2005). Contrary to other reports found in literature, in which a decrease in sperm motility from alcohol-treated animals is usually described (Condorelli *et al*. 2015), in the present model, ethanol ingestion was not able to reduce sperm motility. However, in treated males, there was a lower percentage of hyperactivated spermatozoa than in controls at 60 and 120 min of capacitation. Such an observation would indicate that sperm from treated males show alterations in the biochemical and physiological events, leading to hyperactivated motility, probably related to the increased frequency of morphological abnormalities of the flagellum, possibly associated to abnormal axoneme and periaxoneme.

Since tyrosine phosphorylation of different proteins is usually associated with sperm capacitation (Visconti *et al*. 1995, 2011, Bailey *et al*. 2010), we monitored the pattern of tyrosine phosphorylation in control and treated males. Under our experimental conditions, tyrosine phosphorylated proteins were detected in capacitated CF-1 mouse spermatozoa, but there was no evident difference between phosphorylation patterns in control and treated groups.

When acrosomal exocytosis was evaluated following 120 min of capacitation, spontaneous acrosome reaction was lower in spermatozoa from treated males in comparison to control animals. The decrease in spontaneous acrosomal exocytosis persisted at 150 min when spermatozoa were challenged with progesterone to evaluate induced acrosomal exocytosis. This reduced

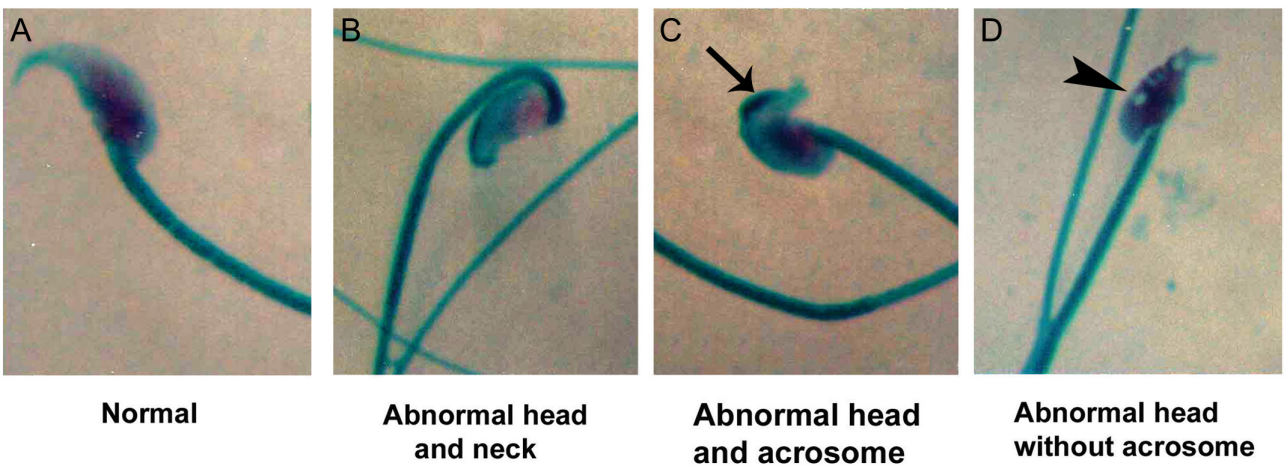


**Figure 6** Morphological sperm abnormalities in CF-1 mouse. (A) Normal head and flagellum. (B) Abnormal head shape with abnormal neck insertion. (C) Abnormal round head with a very small and delocalized abnormal acrosome formation (arrow). (D) Abnormal sperm head with cytoplasm droplet (arrow head) and absence of acrosome. Scale bar: 10 µm.

**Table 2** Frequency of abnormal spermatozoa in control and treated males.

| Groups | **Normal sperm** (mean % ± S.D.M.) | **Abnormal head** (mean % ± S.D.M.) | **Abnormal flagellum** (mean % ± S.D.M.) |
|---|---|---|---|
| Control males ($n=9$) | 84.13 ± 1.63 | 6.9 ± 0.07 | 8.9 ± 0.8 |
| Treated males ($n=9$) | 72.5 ± 2.6*** | 10.5 ± 1.52** | 16.8 ± 2.1*** |

Abnormal spermatozoa from control and treated mice were classified as having amorphous head, neck, and/or midpiece defects and/or an abnormal flagellum. Values are expressed as mean ± S.D.M.
**$P<0.01$, ***$P<0.001$, vs control males, Student's $t$ test.

acrosomal reaction could be indicative of modifications in the sperm membrane, with possible alteration of progesterone receptors, and/or modification of sperm membrane stability that renders the spermatozoon resistant to the action of progesterone. Ethanol can interfere with membrane permeability by disturbing lipid fluidity due to direct oxidation of proteins (Christova *et al*. 2004), and changes in sperm cholesterol content, among other factors, are known to be involved in capacitation (Florman & Ducibella 2006, Bailey *et al*. 2010, Evans *et al*. 2012). The short-term moderate ethanol exposure in these experiments could be inducing similar molecular alterations in the sperm head that could in turn result in a reduced acrosome reaction. Furthermore, we believe that both reduced acrosomal exocytosis and increased percentage of morphologically abnormal sperm heads are alcohol-associated effects in treated mice.

Thus far, our results suggested that sub-chronic moderate ethanol ingestion affected two crucial events prior to fertilization, hyperactivation and acrosomal exocytosis, and also sperm head morphology. It was tempting to hypothesize that this ethanol ingestion paradigm could also alter later events in fertilization, such as ZP penetration, sperm adhesion and fusion to the oolemma, oocyte penetration, and even sperm nuclear decondensation and male pronucleus formation in the ooplasm. Spermatozoa from sub-chronic-ethanol-treated males showed an increased fertilization rate, from the earliest time point examined up to 4.5 h following *in vitro* insemination. Previous reports suggested that ethanol exposure in males negatively affects fertility parameters through the inhibition of capacitation and acrosomal exocytosis (Anderson *et al*. 1983, Rossi *et al*. 2011, Nicolau *et al*. 2014, Wdowiak *et al*. 2014) or by alteration of both *in vitro* and *in vivo* fertilization (Cebral *et al*. 1997). In this study, moderate ethanol administration for 15 days to CF-1 mice produced an increase in the number of oocytes with a decondensed sperm head at 2.5 h of insemination which descended abruptly at 3.5 h of IVF when the decondensed nucleus developed into the male pronucleus. These differences in nuclear decondensation kinetics during IVF between control and treated males suggest an acceleration of the process of sperm nuclear decondensation and formation of the male pronucleus in mice sub-chronically treated with 15% ethanol.

Sperm head decondensation seems to be one of main fertility parameters affected in treated males. Differences found in sperm head decondensation kinetics during IVF between treated males and controls led us to analyze the possible effects of ethanol exposure on *in vitro* sperm decondensation of capacitated spermatozoa in the presence of GSH plus heparin. Percentage of decondensed sperm was higher in treated males compared to controls, suggesting once more that ethanol exposure leads to an acceleration of sperm nuclear decondensation.

Chromatin remodeling, which occurs during differentiation of elongating spermatids and sperm maturation, involves the replacement of histones by protamines, and is a prerequisite for adequate sperm nuclear function and structure. Redox reactions contribute to this process by sulfoxidation of protamines with the participation of glutathione peroxidase (GPX), enzyme that specifically catalyzes the detoxification of hydrogen peroxide. This enzyme, which is present at a considerable quantity in the sperm midpiece, is the main contributor to sperm chromatin stability and the maintenance of mitochondrial membrane potential. Spermatozoa of mice lacking sperm nuclear GPX activity display an abnormal nucleus, showing delayed and/or defective nuclear compaction, nuclear instability and DNA damage. Sperm from nuclear GPX4-knockout mice are more prone to decondense during epididymal maturation than those from wild-type mice and also show significant reductions in forward motility and mitochondrial membrane potential and a structurally abnormal flagellum at the midpiece with swelling of the mitochondria (Fujii & Imai 2014). Given that the present ethanol treatment resulted in an increase in morphologically abnormal spermatozoa, decreased hyperactivation and acrosomal reaction and dysregulation of sperm head decondensation kinetics, we propose that ethanol exposure is hindering the expression and/or activity of GPX in the developing ethanol-exposed spermatozoon. Our laboratory has recently reported an increase in reactive oxygen species and oxidative stress, similar to a high oxidative status, in reproductive-gestational tissues following alcohol exposure (Coll *et al*. 2018). A similar effect could be also induced by ethanol exposure during sperm development/maturation in the testis and epididymis and thus contribute to altered sperm compaction. Our present findings go along with previous reports in the literature stating that ethanol consumption produced spermatozoa with a less compacted chromatin (Talebi *et al*. 2011), and induced abnormalities in external and acrosomal membranes, in chromatin packaging and

altered the nuclear integrity of spermatozoa (Eid *et al*. 2002, Lewis-Jones *et al*. 2003, Gaur *et al*. 2010, Cebral *et al*. 2011, Joo *et al*. 2012, Anifandis *et al*. 2014).

Taken together, the results presented in this paper suggest that sub-chronic ingestion of alcohol negatively affects sperm morphology, capacitation parameters and IVF dynamics. The identification of changes in sperm morphology and function and fertilization events, following short-term ethanol consumption in the male mouse, will allow us to develop new studies aimed at understanding the mechanisms underlying the short-term ethanol-induced sperm effects and their consequences on early embryo development.

## Declaration of interest

The authors have no conflict of interest. All co-authors have read, approved and concur with the submitted manuscript. The authors have ensured the integrity of the work. There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. There is no potential conflict of interest with any financial aid.

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## Author contribution statement

M C S was involved in experimental design and procedures, data analysis and interpretation. V F and C S played a role in experimental procedures and manuscript revision. C G and M Y C were involved in the phosphotyrosine experiments. L C took part in data analysis and interpretation as well as in the final revision of the manuscript, J C C and E C were involved in concept and experimental design, data analysis, interpretation, article draft and revision of manuscript.

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