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ARTICLE

N-Acetyl cysteine: could it be an effective adjuvant therapy in ICSI cycles? A preliminary study

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Abstract This randomized controlled trial tested the hypothesis that addition of *N*-acetyl cysteine (NAC) can increase the probability of pregnancy in intracytoplasmic sperm injection (ICSI) cycles using the long agonist protocol. Women undergoing ICSI cycles due to male factor were randomly assigned to receive either long protocol (group A, 38 women) or long protocol plus NAC (group B, 38 women). Clinical pregnancy was the primary outcome. Granulosa cell apoptosis, fertilization rate, number of grade-one embryos and ongoing pregnancy were the secondary outcomes. Clinical pregnancy rate was insignificantly higher in NAC group (52.6%) than control (47.4%). Early and late apoptosis were also insignificantly lower in group B than in group A. Irrespective of the used protocol, there was significant negative correlation between both early and late apoptosis and fertilization rate (both $P < 0.001$) and the number of good-quality embryos ($P = 0.007$ and $P < 0.001$, respectively). Pregnant patients had significantly lower early and late apoptosis than those who didn't achieve pregnancy ($P < 0.001$). In conclusion, NAC supplementation did not significantly increase the probability of pregnancy in ICSI cycles using long agonist protocol. It appears that granulosa cell apoptosis may be an important prognosticator for ICSI cycle outcome.

KEYWORDS: apoptosis, assisted reproductive techniques, human granulosa cells, infertility, *N*-acetyl cysteine

Introduction

The extensive evidence supporting the long gonadotrophin-releasing hormone (GnRH) agonist protocol has led to its widespread adoption as the standard protocol for ovarian stimulation in IVF and intracytoplasmic sperm injection (ICSI) cycles. However, there is a current growing tendency toward the introduction of GnRH antagonists with many suggested advantages. Still, the meta-analysis of five large randomized trials comparing daily GnRH antagonist injections

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with long GnRH agonist protocols in IVF patients showed an overall decrement in pregnancy rate of 5% in the antagonist groups (Al-Inany and Aboulghar, 2002). A recent Cochrane Database Systematic Review concluded that GnRH antagonist protocol is a short and simple protocol with significant reduction in incidence of severe ovarian hyperstimulation syndrome (OHSS) and amount of gonadotrophins. However, clinical pregnancy rate and ongoing pregnancy/live-birth rate were significantly lower in the antagonist group compared with the long protocol of GnRH agonist (Al-Inany et al., 2006).

Trials to improve the efficacy of ovarian stimulation never stop. The introduction of antioxidant to ovarian stimulation therapy has proved to have many benefits regarding cell mitosis and apoptosis, since oxidants are known to induce cell apoptosis. There have been studies that antioxidants inhibit cell apoptosis of granulosa cells (GC) (Pias and Aw, 2002).

N-Acetyl-cysteine (NAC) is an acetylated form of the amino acid L-cysteine. NAC is a powerful antioxidant, a premier antitoxin and immune support substance. The key to the antioxidant power of NAC is due to its role as a precursor of glutathione, which is one of the most important naturally occurring antioxidants (Ben-Ari et al., 2000; Wentzel et al., 1997). In addition, NAC was found to inhibit apoptosis in cultured ovarian primordial germ cells (Lee et al., 2000). Absence of reported side effects makes NAC an attractive target for research (Fulghesu et al., 2002).

Grundker and Emons (2003) had shown that GnRH1 and GnRH2 receptor activation in the ovary may result in decreased apoptosis. On the contrary, a pro-apoptotic effect of GnRH agonists has been suggested by several lines of research showing the ability of these agents to: (i) increase the incidence of apoptosis in porcine and human cultured GC (Zhao et al., 2000); (ii) decrease in-vitro human luteinized GC proliferation (Matsubara et al., 2000); and (iii) act in-vivo as ovarian atretogenic factors (Billig et al., 1994).

Remarkably, the incidence of GC apoptosis has been related to assisted reproduction outcome. A high level of apoptosis in human cumulus cells may cause poor development of oocytes as well as embryos (Nakahara et al., 1997a,b). *N*-Acetyl cysteine is a powerful antioxidant, anti-apoptotic, with many ovulation-enhancing effects. Therefore, this study queried whether NAC addition, to the standard long protocol could be beneficial.

An intensive PubMed search did not reveal any studies on the addition of NAC to ovarian stimulation in ICSI cycles using the long agonist protocol. So, the aim of the current preliminary study was to test the hypothesis that the addition of NAC to ovarian stimulation can increase the probability of pregnancy — possibly through its effects on GC apoptosis, fertilization rate and embryo quality — in women undergoing ICSI cycles using the long agonist protocol.

Materials and methods

Participants

This randomized controlled trial was conducted during the period from September 2006 to September 2007, following approval by the institutional review board at Zagazig University School of Medicine. An appropriate sample size for such a study was calculated as approximately 200 women in each arm. As this was a self-funded preliminary study, the study was limited to a smaller sample size. The number of cases was chosen to be similar to the number managed during the year prior to the study (June 2005 to June 2006), which was 75 cases. Thus 38 cases were included in each group. Women enrolled for ICSI cycles, due to male factor infertility, were counselled for inclusion in the study. The inclusion criteria were age $\leq$37 years, a body mass index (BMI) of 18—29 kg/m^2, regular menstrual cycles every 24—35 days, presence of both ovaries, day-3 serum FSH $<$9.5 IU/l and basal total antral follicle count greater than six. An informed consent was obtained from all couples who participated in the study.

Randomization

Before starting ovarian stimulation, participants were randomized into two groups, 38 women each, using block randomization technique. Group A was assigned to take the standard long protocol and group B was assigned to take standard long protocol plus NAC (Sedico, Cairo, Egypt), 1200 mg daily in three divided doses, starting from the first day of human menopausal gonadotrophin (HMG) administration till the day of human chorionic gonadotrophic. Thus, 76 identical sealed envelopes were prepared by one of the authors (MIM) and kept in the unit pharmacy. When the woman was eligible and agreed to participate, she was instructed to select one envelope, only once, to determine the group to which she was assigned. The randomization key was kept with the pharmacy director and was not opened until after statistical analysis. Neither randomization concealment nor blinding were possible as an NAC placebo could not be used.

Stimulation regimen

In all participants, the long luteal down-regulation protocol was used. Briefly, GnRH agonist triptorelin (Decapeptyl; Ferring, Kiel, Germany) 0.1 mg was daily administered subcutaneously from the midluteal phase of the pre-treatment cycle. After satisfactory pituitary desensitization was achieved (endometrium $<$5 mm, no ovarian cysts or any follicle $<$10 mm and serum oestradiol $<$50 pg/ml, conversion factor = 3.671 pmol/l), the dose of GnRH agonist was reduced to 0.05 mg/day and ovarian stimulation was initiated with recombinant FSH (Puregon; Organon, Netherlands) and HMG (Menogon). Thereafter, a changing dosage of gonadotrophin was given from day 6 of stimulation according to sequential transvaginal ultrasonography and serum oestradiol. When at least three follicles had reached $\geq$17 mm in diameter, HCG (Pregnyl; Organon) 10,000 IU was administered as a single i.m. dose.

In all cases, oocytes were retrieved by transvaginal ultrasound-guided follicular aspiration 35 hours after HCG injection. Determination of granulosa cell apoptosis was done in all cases, on retrieval day, using annexin—propidium iodide in flow cytometry. ICSI was performed in a standard way. Embryo transfer was performed 2 days later.

Luteal phase support

Daily intramuscular injection of progesterone (Gestone; Pains and Byrne Limited, Surrey, United Kingdom) 100 mg started from the day of embryo transfer and continued until a negative pregnancy test or a positive fetal heart beat was documented by transvaginal ultrasound. In all groups, serum HCG tests were performed on days 16 and 18 after the administration of HCG. An ultrasound scan was done 3 weeks after a positive pregnancy test to confirm a clinical pregnancy. Ongoing pregnancy was defined as pregnancy developing beyond 12 weeks gestation.

Hormone measurements

Serum concentrations of FSH, LH, oestradiol, progesterone and βHCG were determined using Elecsys 2010 (Roche, Germany). For FSH, the analytical sensitivity was <0.1 IU/l with total precision of 2.9%. For LH, the analytical sensitivity was 0.1 IU/l with total precision of 1.6%. For oestradiol, the analytical sensitivity was 5 pg/ml with total precision of 2.3%. For progesterone, the analytical sensitivity was 0.03 ng/ml (conversion factor = 3.18 nmol/l) with total precision of 2.4%. For quantitative βHCG assay, the analytical sensitivity was 0.5 IU/l with total precision of 2.1%.

Flow cytometric assessment of GC apoptosis

GC isolation

After isolation of the oocytes, all follicular fluid from each woman were pooled in a tube to have sufficient material for analysis and the tubes were left standing for 5 min to allow the GC to sediment. The GC were then transferred to a tube containing 5 ml of Ham's F-10 medium (Sigma–Aldrich, Milano, Italy) and gently re-suspended. After centrifugation at 1300*g* for 10 min, GC were re-suspended in 5 ml of Ham's F-10 medium. To prevent cell aggregation, GC were treated with hyaluronidase solution (0.1% v/w in Ham's F-10) for 10 min. The cells were mechanically dispersed with repeated aspiration and expiration through a fine Pasteur glass pipette. Then, the cells were re-suspended in phosphate-buffered saline without calcium and magnesium. The entire procedure was completed within an hour after follicle aspiration to prevent cell death.

Determination of GC apoptosis (annexin V–propidium iodide detection flow cytometry)

Early apoptotic cells are recognizable through reversible binding of annexin V to phosphatidyl serine, a membrane phospholipid that is exposed at the beginning of the apoptotic process (Fadok et al., 1992). Purified GC were assessed for apoptosis, in parallel with positive controls. For positive control, the cells were incubated with 3% formaldehyde in buffer during 30 min on ice. Formaldehyde was then washed away and the cells were suspended in cold diluted binding buffer at 10^5–10^6 cells/ml. A commercial kit (Annexin V–FITC Kit; Nexins Research, Kattendijle, Netherlands) was used according to the manufacturer's instructions. Briefly, the cells were gently re-suspended in binding buffer and incubated for 10 min at room temperature in the dark with annexin V–FITC. Samples were then washed and supravitally stained with propidium iodide (PI, 50 μg/ml), a membrane impermeable stain, that allows the discrimination between membranes altered necrotic (bright) and apoptotic (dim) cells. All samples were measured by BD FACSort flow cytometer with the FL3 detector (Becton Dickinson, USA), in a log mode with an argon laser at 488 nm for excitation. In all cases, data were collected on 10^4 viable cells, by electronic gating on forward and side scatter light parameters. For comparative staining, we used the mean fluorescence intensity (MFI), which was defined as the average fluorescence value of the corresponding staining referred to the logarithmic scale of fluorescence intensity, along the *X*-axis of histograms. Vital cells were annexin V negative/PI negative, early apoptotic cells were annexin V positive/PI negative and necrotic cells were annexin V positive/PI positive.

Outcome measures

Clinical pregnancy was the primary outcome. GC apoptosis, fertilization rate, number of grade-one embryos and ongoing pregnancy were the secondary outcome measures.

Statistical analysis

Data were statistically described in terms of mean ± SD, median, frequencies (number of cases) and relative frequencies (percentages) when appropriate. Analysis was done in accordance with an intention to treat. Comparison of quantitative variables between the study groups was done using Mann–Whitney *U*-test for independent samples. For comparing categorical data, chi-squared test was performed. Exact test, was used instead when the expected frequency was less than 5. Correlation between various variables was done using Pearson moment correlation equation, for linear relation in normally distributed variables. Multivariate analyses were done to test the effect of NAC addition on early and late apoptosis, number of grade-one embryos and fertilization rate, as well as the association between apoptosis and clinical pregnancy after adjusting for all important factors (age, BMI, duration of infertility, basal FSH, basal antral follicle count, endometrial thickness, serum oestradiol on HCG day, serum progesterone on HCG day and number of HMG ampoules). A probability value less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Excel version 7 (Microsoft Corporation, NY, USA) and SPSS version 15 (SPSS, Chicago, IL, USA).

Results

A total of 200 patients were potentially eligible for recruitment. Forty-two women refused to participate, 70 did not meet the inclusion criteria and 12 were excluded for other reasons. Therefore, after down-regulation, with the start of ovarian stimulation, 76 women were randomized into group A (standard long protocol, $n = 38$) and group B (standard long protocol plus NAC, $n = 38$) (**Figure 1**). During HMG stimulation, three cases were cancelled due to impaired response (fewer than three follicles during ovarian stimulation), two cases in group A and one case in group B. These three cases were included in the intention-to-treat

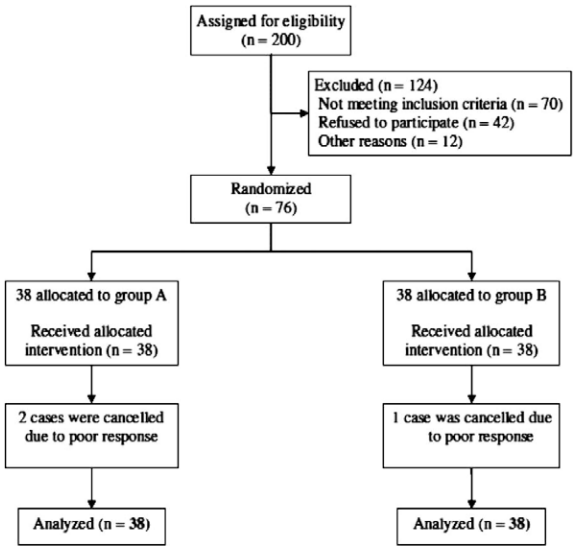

Figure 1 Flowchart of participants in the study.

analyses. The two groups were balanced in respect of demographic, clinical and ICSI cycles characteristics. No differences were found regarding age, duration of infertility, BMI, basal FSH and mean antral follicle count (Table 1). Number of ampoules, days of stimulation, oestradiol and progesterone concentrations on day of HCG and endometrial thickness were comparable between the two groups. Retrieved, mature and fertilized oocytes showed a trend towards higher numbers in the NAC group, but none reached statistical significance. Similarly, the number of cleaved and grade-one embryos were tended towards higher values in the NAC group, but neither were significantly higher. The number of transferred embryos was also comparable between the two groups (Table 2).

Clinical and ongoing pregnancy rates were higher in group B (20/38, 52.6%, 95% CI 36.72 to 68.48; 18/38, 47.4%, 95% CI 31.52 to 63.28, respectively) than group A (18/38, 47.4%, 95% CI 31.52 to 63.28; 16/38, 42.1%, 95% CI 26.4 to 57.8, respectively), but differences were not statistically significant. The relative risks of clinical and ongoing pregnancy between group A and group B were 1.111 (95% CI 0.708 to 1.744) and 1.125 (95% CI 0.681 to 1.857), respectively. In addition, there was no statistically significant difference between the two groups regarding the occurrence of miscarriage. NAC was well tolerated by all participants with no reported side effects.

Table 1 Participants' characteristics in the two ovarian stimulation groups.

Characteristic	*Long protocol (group A, n = 38)*	*Long protocol + NAC (group B, n = 38)*
Age (years)	27.97 ± 3.77	26.37 ± 4.05
BMI (kg/m^2)	27.24 ± 1.29	27.15 ± 1.92
Duration of infertility (years)	6.89 ± 3.61	6.75 ± 3.81
Basal FSH (IU/l)	6.39 ± 1.21	6.12 ± 1.15
Basal AFC	10.18 ± 2.32	9.95 ± 2.28

Values are mean ± SD.
AFC = antral follicle count; BMI = body mass index; NAC = *N*-acetyl cysteine.
There were no statistically significant differences between the two groups (Mann–Whitney *U*-test).

Table 2 ICSI cycle parameters of the two ovarian stimulation protocols.

Parameter	*Long protocol (group A, n = 38)*	*Long protocol + NAC (group B, n = 38)*
Gonadotrophin ampoules	33.50 ± 6.07	32.79 ± 8.08
Duration of stimulation (days)	10.66 ± 1.12	10.87 ± 0.87
Serum oestradiol on HCG day (pg/ml)	2569.6 ± 696	2308.4 ± 672
Serum progesterone on HCG day (ng/ml)	0.91 ± 0.65	1.09 ± 0.69
Endometrial thickness (mm)	8.71 ± 1.08	8.92 ± 1.17
Oocytes retrieved	11.87 ± 5.72	13.79 ± 4.99
Mature oocytes	9.32 ± 4.35	10.45 ± 4.79
Fertilized oocytes (2PN)	6.84 ± 3.46	7.89 ± 3.48
Fertilization rate (%)	70.69 ± 10.44	72.17 ± 12.32
Cleaved embryos	5.82 ± 2.51	6.47 ± 2.52
Grade-one embryos	4.34 ± 1.83	4.95 ± 1.75
Embryo transfers	2.68 ± 0.98	2.79 ± 1.14

Values are mean ± SD.
HCG = human chorionic gonadotrophin; NAC = *N*-acetyl cysteine.
There were no statistically significant differences between the two groups (Mann–Whitney *U*-test).

GC apoptosis

Both early apoptosis (annexin V positive/PI negative cells) and late apoptosis (annexin V positive/PI negative cells) were lower in group B (median = 1.7% and 2.5%, respectively) than in group A (1.9% and 3.7%, respectively), but the differences were not statistically significant (**Figure 2**).

When multivariate regression analysis was performed, there was no statistically significant effect of adding NAC to the long protocol, neither on early nor late apoptosis. Also, none of the tested variables had a statistically significant effect. Similar analyses were done to test the effects on the number of grade-one embryos and fertilization rate and none had achieved statistical significance.

Irrespective of the protocol, there was significant negative correlation between number of mature oocytes ($r = -0.267$, $P = 0.02$), fertilization rate ($r = -0.492$, $P < 0.001$), number of cleaved embryos ($r = -0.287$, $P = 0.012$), number of grade-one embryos ($r = -0.305$, $P = 0.007$) and the percentage of early apoptosis (**Figure 3**).

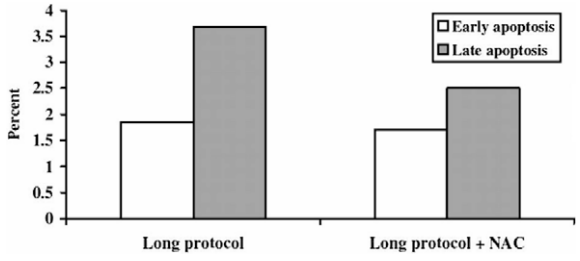

Figure 2 Early and late apoptosis of granulosa cells in the two ovarian stimulation groups.

Similarly, there was significant negative correlation between number of mature oocytes ($r = -0.385$, $P = 0.001$), fertilization rate ($r = -0.504$, $P < 0.001$), number of cleaved embryos ($r = -0.398$, $P < 0.001$), number of grade 1 embryos ($r = -0.399$, $P < 0.001$) and the percentage of late apoptosis (**Figure 4**).

Pregnant patients and patients who didn't get pregnant were balanced in respect of the patients and ICSI cycle characteristics (**Table 3**). Still, pregnant patients had statistically significantly lower both early and late apoptosis (median = 1.13% and 0.86%, respectively) than those who didn't achieve pregnancy (median = 5.9% and 14.4%, respectively, $P < 0.001$). Upon performing multivariate analyses — after adjusting for age, BMI, duration of infertility, basal FSH, basal antral follicle count, endometrial thickness, serum oestradiol on HCG day, serum progesterone on HCG day and number of HMG ampoules — there was a strong statistically significant relationship between clinical pregnancy and both early (**Table 4**) and late apoptosis (**Table 5**) ($P < 0.001$). None of other variables showed statistically significant effect.

Discussion

In the current study, a trial was made to verify the efficiency of treatment with the antioxidant, NAC, during ovarian stimulation, with the standard long protocol. NAC effects were assessed in terms of various cycle parameters, ovarian response, granulosa cell apoptosis and pregnancy rate.

A total of 76 infertile couples, candidate for ICSI cycles were included in the study. Only patients with male factor infertility were included to investigate the actual effect of the drug in absence of any female cause that might have affected the results. Women were randomized to receive either long protocol (group A) or long protocol + NAC (group B).

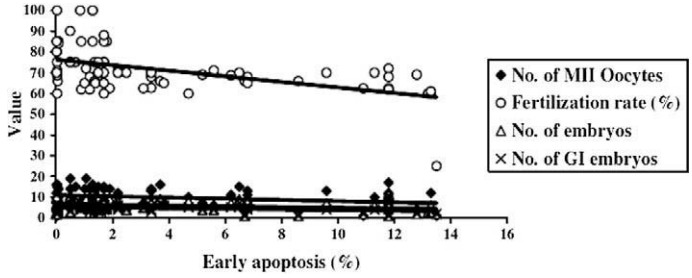

Figure 3 Correlation between early apoptosis and number of metaphase II (MII) oocytes, fertilization rate, number of cleaved and grade-one (G1) embryos.

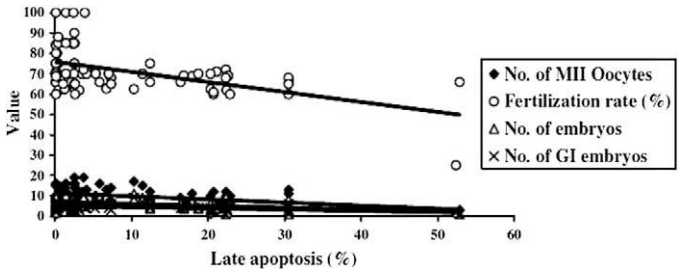

Figure 4 Correlation between late apoptosis and number of metaphase II (MII) oocytes, fertilization rate (%), number of cleaved and grade-one (G1) embryos.

Table 3 Patient and ICSI cycle characteristics of pregnant and non-pregnant patients.

Characteristic	*Pregnant (n = 38)*	*Non-pregnant (n = 38)*
Age (years)	26.37 ± 4.1	27.97 ± 3.75
BMI (kg/m^2)	27.08 ± 1.81	27.32 ± 1.44
Duration of infertility (years)	6.29 ± 3.63	7.36 ± 3.72
Basal FSH (mIU/ml)	5.99 ± 1.1	6.53 ± 1.22
Antral follicle count	9.95 ± 2.11	10.18 ± 2.49
Endometrial thickness (mm)	9.00 ± 1.1	8.63 ± 1.15
Serum oestradiol (pg/ml)	2471.4 ± 631	2406.6 ± 754
Serum progesterone (ng/ml)	1.01 ± 0.70	1.01 ± 0.67
HMG ampoules	34.16 ± 7.51	32.13 ± 6.63
HCG day	10.71 ± 1.16	10.82 ± 0.83
Retrieved oocytes	13.16 ± 5.51	12.50 ± 5.39
MII oocytes	10.87 ± 3.95	8.89 ± 5.0
2PN oocytes	8.13 ± 3.19	6.61 ± 3.66
Fertilization rate	72.76 ± 9.86	70.12 ± 12.70
Embryos	6.66 ± 2.04	5.63 ± 2.86
Grade-1 embryos	4.97 ± 1.24	4.32 ± 2.21
Embryos transferred	2.95 ± 0.66	2.53 ± 1.33
Early apoptosis (%)	1.10 ± 0.98[a]	6.26 ± 4.56[a]
Late apoptosis (%)	2.10 ± 4.08[b]	15.46 ± 13.0[b]

Values are mean ± SD.
BMI = body mass index; HCG = human chorionic gonadotrophin; HMG = human menopausal gonadotrophin; NAC = *N*-acetyl cysteine.
There were no statistically significant differences between the two groups (Mann–Whitney *U*-test) except in early and late apoptosis ([a]$P < 0.001$; [b]$P < 0.001$; Mann–Whitney *U*-test).

Table 4 Multivariate regression analysis for the relationship between clinical pregnancy and early apoptosis adjusted for other variables.

Variable	*Coefficient*	*95% CI*	*P-value*
Ovarian stimulation protocol	−0.028	−1.039 to 1.631	NS
Age	0.130	−0.126 to 0.188	NS
BMI	0.060	−0.447 to 0.093	NS
Duration of infertility	−0.119	−0.602 to 1.109	NS
Basal FSH	0.121	−0.217 to 0.757	NS
Endometrial thickness	−0.159	−0.436 to 0.441	NS
Basal AFC	0.179	−0.152 to 0.277	NS
Serum oestradiol on HCG day	−0.067	−0.001 to 0.001	NS
Serum progesterone on HCG day	0.107	−1.513 to 2.099	NS
HMG ampoules	−0.058	−0.099 to 0.051	NS
HCG day	0.228	−0.179 to 0.025	NS
Clinical pregnancy	−0.574	−6.335 to −3.219	<0.001

AFC = antral follicle count; BMI = body mass index; HCG = human chorionic gonadotrophin; HMG = human menopausal gonadotrophin; NS = not statistically significant.

Patients' characteristics and various cycle parameters were comparable between the two groups. The NAC-associated increase in the number of retrieved, mature and fertilized oocytes did not reach the significant level. Similarly, the increase in the number of cleaved and grade-one embryos in the NAC group did not reach statistical significance. Clinical and ongoing pregnancy rates were higher in group B (52.6% and 47.4%) than in group A (47.4% and 42.1%) but differences were not statistically significant.

The present study tried to verify whether NAC administration had conditioned the level of granulosa cells apoptosis, which might allow optimum synchronization of cytoplasmic and nuclear maturation of the oocyte (Quirk et al., 2004). NAC, a thiol antioxidant, might be a potential inhibitor of apoptosis for various reasons: (i) it is a well-established inhibitor of physiological cell death in several other systems and a compound known to act on GC as a survival factor (Otala et al., 2002); (ii) on the basis of previous studies on human

Table 5 Multivariate regression analysis for the relationship between clinical pregnancy and late apoptosis adjusted for other variables.

Variable	*Coefficient*	*95% CI*	*P-value*
Ovarian stimulation protocol	−0.038	−5.573 to 3.795	NS
Age	0.073	−0.512 to 0.943	NS
BMI	0.003	−1.407 to 1.450	NS
Duration of infertility	−0.211	−1.374 to 0.036	NS
Basal FSH	0.100	−1.131 to 3.101	NS
Endometrial thickness	−0.061	−2.622 to 1.363	NS
Basal AFC	0.171	−0.079 to 1.821	NS
Serum oestradiol on HCG day	−0.105	−0.006 to 0.002	NS
Serum progesterone on HCG day	0.119	−1.340 to 5.470	NS
HMG ampoules	0.051	−0.227 to 0.397	NS
HCG day	0.124	0.061 to 4.633	NS
Clinical pregnancy	−0.539	−16.991 to −8.061	<0.001

AFC = antral follicle count; BMI = body mass index; HCG = human chorionic gonadotrophin; HMG = human menopausal gonadotrophin; NS = not statistically significant.

testis, NAC penetrates the tissue very well and remains effective in cultures (Erkkilä et al., 1998); and (iii) it is a widely used and clinically safe drug (Ahola et al., 1999). Recently, Hung et al. (2009) concluded that NAC reduces oxidative stress, thus preventing high glucose induced mesangial apoptosis. This study's results showed that both early and late apoptosis were lower in NAC group (median = 1.7% and 2.5%, respectively) than in the control group (1.9% and 3.7%, respectively) but with insignificant difference, even after performing multivariate regression analysis. Larger-scale studies have to be conducted to clarify real impact of NAC addition, if it exists.

Moreover, there could be other scenarios. In the current study, 1200 mg NAC was used daily, from the beginning of HMG till the day of HCG. In a study by Otala and colleagues (2002), NAC was found to inhibit apoptosis in ovarian tissue cultures in a dose-dependant manner. Rizk et al. (2005) and Gafaar et al. (2007), used NAC as an adjuvant to ovarian stimulation, in two different models for IVF. In the first study, 1200 mg daily NAC was given from cycle day 3 for 5 days, in addition to clomiphene citrate. In the second study, 1200 mg daily NAC was given from cycle day 2 till the day of HCG, adjuvant to low fixed-dose HMG. Interestingly, Elnasher et al. (2007) used NAC at a dose of 1800 mg daily for 5–6 weeks in treatment of clomiphene citrate-resistant polycystic ovarian disease patients. So, the possibility that NAC administration for longer duration and/or higher dose might inhibit apoptosis and probably affect cycle outcome should be thoroughly investigated.

The activation of apoptosis in the oocyte is regulated by molecular signals coming from cumulus cells through gap junctions. A lower apoptotic rate in cumulus cells is considered an indicator of good oocyte quality, with a greater capacity to be fertilized and produce embryos with a higher implantation potential (Kaneko et al., 2000; Suh et al., 2002). This study's results seem to confirm this scenario. An interrelationship between GC apoptosis and ICSI outcome has been shown, no matter which stimulation protocol is used. There were negative correlations between the number of mature oocytes, fertilization rate and the number of cleaved/good-quality embryos and the incidence of early and late apoptosis. Moreover, there was a strong statistically significant relation between clinical pregnancy and both early and late apoptosis ($P < 0.001$), both in the univariate and multivariate analyses with adjustment of other important variables.

Clavero et al. (2003) reported that GC in IVF cycles require physiologic spermatozoon–oocyte interactions that only succeed with a good-quality oocyte (i.e. one with less GC apoptosis). However, they suggested that the invasive nature of ICSI bypasses these natural barriers, thus does not require a good-quality oocyte. On the contrary, Bosco et al. (2005) demonstrated that high apoptosis in human oocytes determines fertilization failure after ICSI.

In the current study, GC apoptosis was detected with annexin V/PI with flow cytometry. This technique offers the possibility of a multiparametric analysis of cellular attributes, including asymmetry of the plasma membrane and DNA fragmentation. This permits evaluation and discrimination between early and late phases of apoptosis. Moreover, measuring apoptosis in flow cytometry increases the outcome precision and allows the analysis of far more GC in a short time. In fact, the externalization of phosphatidyl serine at the cell plasma membrane level – a very early phenomenon during apoptosis – can be detected by virtue of its affinity for annexin V (Fadok et al., 1992).

The levels of apoptosis found in this study seem to be higher than those found by other investigators (Nakahara et al., 1997a,b). This inconsistency could be explained by the different sensitivities of the assays used for characterizing apoptosis. Nakahara et al. (1997a,b) measured the percentage of GC apoptosis by using fluorescence microscopy. Only the apoptotic bodies (indicative of late apoptosis) of up to 1000 granulosa-lutein cells were counted with the use of fluorescence microscopy. Meanwhile, measuring apoptosis by flow cytometry has the advantage of allowing the analysis of >50,000 cells in a short time (Nakahara et al., 1997a,b; Saito et al., 2000). This study's results coincide with those obtained by Clavero et al. (2003) and Oosterhuis et al. (1998) who also used flow cytometry to measure the percentage of apoptotic GC.

In contrast, Giampietro et al. (2006) used the same technique as in the present study but reported a higher

percentage of apoptosis. However, the present study's participants were healthy women (male factor only), while Giampietro et al. (2006) studied women with different causes of female factor infertility.

As far as is known, this is the first trial of NAC addition to ovarian stimulation in ICSI cycles using the long agonist protocol. The current preliminary study included 76 cases, $\leq$37 years of age with male factor infertility. Larger-scale studies have to be definitely conducted for further evaluation.

In conclusion, this study found that 1200 mg NAC supplementation starting with the use of HMG till the day of HCG did not significantly increase the probability of pregnancy in ICSI cycles using the long agonist protocol. NAC treatment was associated with insignificant decrease in granulosa cell apoptosis, as well as insignificant increase in fertilization rate and grade-one embryos. Larger-scale studies, possibly with higher doses and or longer duration of NAC use, should be performed to identify any significant effects. It appears that GC apoptosis may be an important prognosticator for ICSI cycles outcome.

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