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GAMETE BIOLOGY

Importance of sperm gluthatione treatment in ART

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Abstract
Purpose The aim of this study was to investigate whether treatment of sperm from infertile patients would gluthatione could reduce sperm premature chromosome condensation (PCC). To reach the goals of this study, the frequency of sperm PCC formation in sperm of normal and sub-fertile men with/without glutathione treatment were compared and analyzed.
Methods Hamster oocytes were retrieved after super ovulation by PMSG and HCG injection. Following treatment with hyaluronidase, zonae was removed by trypsin digestion. Sperm were classified into 3 groups according to morphology, movement and counts, treated with glutathione(10 μg/ml) and then processed by swim up method. After capacitation, zona-free oocytes were incubated with sperm then transferred to fresh media containing colcemid. Cells were fixed and slides prepared using Tarkowskie's standard air drying technique and after staining in 5% Giemsa, oocytes were analyzed at high magnification.
Results Sperm penetration rate was higher and the rate of intact sperm head and PCC was lower in GSH treated samples compared to non treated groups. Sperm penetration rate was significantly higher in treated astheno sperm samples compared to non-treated ones (66.4% and 50. 97% respectively) ($P<0.001$). We observed a significantly higher PCC frequency in infertile patients ($P<0.001$). In addition, there was a significantly lower rate of intact sperm head in treated astheno sperm samples (17.49%) compared to non treated ones (26.79%) ($P<0.001$). Finally, a significantly lower rate of PCC in treated astheno sperm samples comparing to non treated ones was seen (51.06% and 72.96% respectively) ($P<0.001$).
Conclusions Our results show that sperm PCC formation could be one of the major causes of failed fertilization in individuals with sperm abnormalities. Also sperm PCC formation may be involved in the etiology of some cases of idiopathic infertility. Given that the susceptibility of sperm to oxidative stress is significantly greater in idiopathic infertile men, our results show that treatment with glutathione could significantly reduce these stress factors and increase ART outcome.

Keywords Human sperm · Premature Chromosome Condensation (PCC) · Glutathione · Male infertility

Capsule A clinical need exists for establishment of approaches which can be utilized to increase the ART success rate by treatment of the infertile patient's sperms with gluthatione.

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Introduction

Male infertility has been related to multiple irregularities including sperm concentration, motility and morphology [7, 8]. Oocyte immaturity has been considered as an underlying factor in induction of PCC [2]. In this study all hamster oocytes were collected at MII phase Rosenbuch suggested that sperm chromatin anomalies can contribute to induce sperm

PCC [9]. Therefore, the aim of this study was to investigate the effect of gluthatione treatment on inhibition of PCC in human sperm co-incubated with hamster oocytes. When a cell, whose chromosomes are in metaphase, is fused with an inter-phase cell, the nuclear membrane of the cell in inter-phase dissolves and its chromatin condenses. This phenomenon is called premature chromosomal condensation [1]. In mammalian sperm, protamines serve to stabilize the nucleus and maintain the DNA in a transcriptionally inert condition. Protamines contain numerous free sulfhydryls. During maturation of sperm in the epididymis these protamine sulfhydryls undergo oxidation, resulting in further stabilization of the sperm nucleus [2]. Following penetration of sperm into an oocyte; oocyte activation is triggered, resulting in completion of meiosis and formation of both male and female pronuclei [1]. Protamine must be reduced during fertilization so that reactivation of the sperm nucleus will proceed. This disulfide bond reduction begins shortly after the sperm nucleus makes contact with the ooplasm and it is a prerequisite for sperm chromatin dispersion or decondensation, and hence for male pronucleus formation [2]. Under some circumstances although the sperm is within the oocyte, fertilization fails to occur. In this condition, the oocyte remains in the metaphase II (MII) stage while the sperm head transforms into PCC separate from the oocyte chromosomes. In this situation the oocyte fails to become activated and remains arrested at MII and because of the presence of meiosis promoting factor (MPF), sperm chromatin is transformed into condensed chromatin [3–5]. However, glutathione (GSH)," the major intracellular free thiol has been implicated as playing an important role in these events [2]. Glutathione which is a widely distributed thiol in animal organisms is involved in the protection of male and female gametes against oxidative damages. Furthermore, GSH concentrations in the maturing spermatozoa gradually decline during spermatogenesis [6]. It has been implicated in maintaining the meiotic spindle morphology of the oocyte. After fertilization, this thiol plays an active role in the formation of the male pronucleus, and has a beneficial effect on early embryogenesis to the blastocyst stage.

Materials and methods

Media

Hams-F10 medium (Biochrom.K.G) with some modifications is used for sperm preparation, capacitation, egg preparation and IVF. These modifications include adding 2104mgr/lit $NaHCO_3$ and 248mgr/lit Calcium Lactate to the medium. This media was supplemented with 15% fetal bovine serum (Sigma). Media was incubated in 5% CO2 and37C. (The buffer in the media will lower the pH slightly and keep it on 7.4 at 37 C).

Semen sample preparation and gluthatione treatment

Ninety phenotypically normal non-smoker individuals who referred to Royan Institute were included in the study. None of these patients have received any antibiotic or prior drug treatment for the last 2 months before their referral to our clinic. Adult patients were selected regardless of race or age (mean age of 32.46±6.96). The semen samples were collected in sterile containers (Falcon) and processed after liquification (10–20 min at 37 C). Total sperm counts, number of sperms/ml, motility and forward progression were determined for each sample. Sperm samples divided into three groups according to WHO criteria (Normal, Oligosperm, and Asthenosperms). For each group thirty (30) samples were studied. Sperms were processed by Swim-Up technique, were treated and incubated with 10 mM gluthatione for three (3) hours in Hams-F10 medium in 37 C CO2 incubator for capacitation. The concentration of gluthatione was estimated according to previous different studies of Perreault *et al.,* 1988 and Zuelke *et al.*, 2003 [2, 10]. The capacitation time for normal and oligosperm groups were three (3) hours but asthenosperms samples needed more time comparing to other groups (2 h more). For these patients' gluthatione added to the media 2 h after starting the capacitation process.

Hamster care and super ovulation

Female golden hamsters purchased from Pastor Institute (Tehran-Iran). They were transferred to animal house facility of the laboratory at five to seven (5–7) weeks of age. Hamster super ovulation and oocyte retrieval were done according to standard protocol [11]. Each hamster yields approximately twenty to twenty five (25–50) eggs.

Egg preparation and culture

The hamsters were sacrificed by cervical dislocation and the oviducts were punctured. The cumulus cell mass was removed and transferred to a solution of 0.1% hyaloronidase (Sigma). Oocytes were washed in fresh media and were transferred to a solution of 0.1% trypsin (Gibco) for zona removal. Subsequently, the oocytes were washed in HamsF-10 solution and transferred to fertilization drops containing capacitated sperms at a concentration of 10^6–10^7 per ml of HamsF-10 medium. In order to have a maximum fertilization, the samples were incubated for approximately three (3) hours (for asthenosperm group) to allow sperm penetration to occur. After three (3) hours co-incubation of sperms and oocytes, the eggs were washed two times in HamsF-10 to remove excess sperms. In the next step, we scattered twenty to thirty (20–30) eggs throughout one drop of media and incubated the eggs in CO2 incubator at 37 C overnight. The

next morning the eggs were transferred directly to drops of HamsF-10 solution containing 0.4 μg/ml colcemid (Sigma) under sterile paraffin oil for four to seven (4–7) hours.

Slide preparation

Approximately fifteen (15) eggs were transferred from colcemid solution to a hypotonic solution (1% sodium citrate) for ten (10) min. Subsequently, three to five (3–5) eggs were transferred to the center of a microscope glass slide which were fixed using Tarkowskie's air drying technique [12].

Statistical analysis

All statistical analyses were carried out using SPSS (SPSS Inc., Chicago, Illinois, USA) software version 15. To compare and analyze the results of sperm penetration, intact sperm head and PCC formation in all groups, the $\chi 2$ test was used. P-value of less than 0.05 was considered significant. Odds ratio (OR) and 95% confidence interval (95% CIs) was also calculated for data analysis.

Results

A total of three thousand four hundred seven (3407) zona-free golden hamster oocytes were included in our study. The results are summarized in Tables 1, 2 and 3.

Non-treated groups

In non treated control samples, out of 450 analyzed normal oocytes group; 381 oocytes showed sperm penetration (84.66%). In this group we observed 74 oocytes with sperm PCC (19.42%). In order to obtain more penetration rate and study the adequate number of samples (30 people for each group); the higher numbers of oocytes were analyzed in oligosperm and asthenosperm groups. For oligosperm men 760 oocytes and for asthenosperms 820 oocytes were studied.

In non treated oligosperm group; sperms penetration were seen in 541 oocytes (71.18%) but 193 showed sperm PCC formation (35.67%).

Out of the total analyzed number of 820 oocytes in non treated asthenosperm samples 418 oocytes showed sperm penetration (50.97%). Furthermore, out of 418 oocytes in non treated asthenosperms samples, 305 oocytes showed PCC of sperms (72.96%).

In addition, in non treated control samples ,the statistical analysis showed a significant difference between the penetration rates of normal, oligosperm and asthenosperm ($P<0.001$) (Table 1).

As seen in Table 2, the frequency of intact sperm heads in normal individuals was 8.92%, in oligosperm samples 22.18% and in asthenosperm persons was 26.79%. Statistical analysis showed significant differences between the rate of intact sperm heads in normal versus oligo and astheno persons. The frequency of PCC in normal samples was seen to be the lowest between these three groups and the rate of PCC in asthenosperm group was completely higher than both normal and oligosperm samples. The differences of frequencies between these groups were statistically significant from each other ($P<0.001$) (Table 3).

Treated groups

Among the glutathione treated samples incubated with normal sperm, 307 oocytes were studied. 262 oocytes showed the evidence of sperm penetration (85.34%), of which 45 oocytes were observed with PCC of sperms (17.17%).

Among the glutathione treated sperms obtained from oligosperm men, 433 oocytes were studied. Sperm penetration was seen in 316 oocytes (72.97%), of which 95 showed sperm PCC formation (30.06%).

Among the glutathione treated sperms obtained from asthenosperm men, out of the total 637 oocytes; 423 oocytes showed the sperm penetration (66.4%), of which 216 oocytes showed sperm PCC (51.06%).

Gluthatione treated sperms exhibited higher penetration rate in all groups (Table 1). According to our statistical

Table 1 Results of sperm penetration into zona-free hamster oocytes with/without 10 mM gluthatione treatment

Groups	Control	OR[a](95% CI)	Treated with Gluthatione	OR[a] (95% CI)	P-Value[b]	OR[a] (95% CI)
Normal	84.66% (381/450)	1[c]	85.34%(262/307)	1[c]	0.799	0.95(0.63-1.43)
Oligosperm	71.18% (541/760)	0.45(0.33–0.60)	72.97%(316/433)	0.46(0.32–0.68)	0.507	0.92(0.70–1.19)
Asthenosperm	50.97% (418/820)	0.19(0.14–0.25)	66.4%(423/637)	0.34(0.24–0.49)	<0.001[d]	0.53(0.43–0.65)

[a] Odds Ratio

[b] P-Value for Compare before and after Gluthatione treatment

[c] Reference category

[d] Statistically Different

Table 2 Results of intact sperm head in zona-free hamster oocytes with/without 10 mM gluthatione treatment

Groups	Control	OR[a] (95% CI)	Treated with Gluthatione	OR[a] (95% CI)	P-Value[b]	OR[a] (95% CI)
Normal	8.92%(34/381)	1[c]	7.25%(19/262)	1[c]	0.449	1.25(0.70–2.25)
Oligosperm	22.18%(120/541)	2.91(1.94–4.37)	18.67%(59/316)	2.94(1.70–5.07)	0.223	1.24(0.88–1.76)
Asthenosperm	26.79%(112/418)	3.74(2.47–5.65)	17.49%(74/423)	2.71(1.60–4.61)	<0.001[d]	1.73(1.24–2.40)

[a] Odds Ratio

[b] P-Value for Comparing before and after Gluthatione treatment

[c] Reference category

[d] Statistically Different

analysis; there was a significant difference between the penetration rates of treated and non treated asthenosperm samples ($P<0.001$). Although this parameter was higher in treated oligosperm and normal samples than non treated groups, but these failed to reach statistical significance ($P>0.05$) (Table 1). Intact sperm head and PCC formation was also lower in treated samples comparing with non treated samples, but only in asthenosperm samples it was significantly lower ($P<0.001$) (Tables 1 and 3). Moreover, the rate of PCC and intact sperm head decreased in all groups (Tables 2 and 3). Interestingly the PCC rate in glutathione treated asthenosperm samples was lower than treated oligosperms. The frequency of PCC in normal glutathione treated samples was seen to be the lowest between all 6 groups (Table 3)

Discussion and conclusion

The presence of sperm nucleus PCC in varying degrees of condensation or the presence of non-activated sperm head in the ooplasm (Tables 2, 3) suggest that the successful introduction of spermatozoa into the ooplasm of a metaphase II oocyte is not sufficient to achieve fertilization . Sperm decondensing activity is maximal in mature, metaphase II oocytes, but minimal or absent in immature, germinal vesicle (GV) oocytes [2]. Immature cytoplasms are believed to make sperms susceptible to high incidence of PCC following the insemination because these immature oocytes are unable to undergo oocyte activation [1]. However, it is already known that PCC formation is affected by so many factors both for oocytes and sperm abnormalities. In this study d hamster zona-free oocytes were use to keep the ooplasmic condition similar and constant for all samples. Therefore, the roles of sperm conditions are the only variable in PCC formation in the related experiments. Furthermore, the ooplasmic factors needed for chromatin decondensation of sperms are similar for all the sperms either normal or asthenospermic individuals.

It is known that the sperm decondensing activity declines after fertilization and is again absent or diminished in pronuclear oocytes. Hamster oocyte GSH levels are indeed higher in mature metaphase II oocytes than in GV or fertilized oocytes. In this study, all hamster oocytes were collected at MII phase [2]. PCC was mainly reported for failed fertilized oocytes following IVF or ICSI procedures. According to the results of our study, it seems that one of the important reasons which can describe idiopathic infertility and fertilization failure in asthenosperm patients is PCC induction. Sperm protamine deficiency could be one of the factors that make sperm prone to PCC [1, 13]. As it was mentioned before, protamines in sperms undergo oxidation in epididymis which leads to sperm nucleus stability. Disulfide bonds reduction, which is necessary for reactivation of the sperm nucleus, begins shortly after the sperm nucleus makes contact with the ooplasm. Little is known about the oocyte factors involved in protamine disulfide bond reduction and subsequent dispersion of sperm chromatin during fertilization [1, 2, 13]. Perreault *et al* (1987)

Table 3 Results of sperm PCC in zona-free hamster oocytes with/without 10 mM gluthatione treatment

Groups	Control	OR[a] (95% CI)	Treated with Gluthatione	OR[a] (95% CI)	P-Value[b]	OR[a] (95% CI)
Normal	19.42% (74/381)	1[c]	17.17%(45/262)	1[c]	0.471	1.16(0.77–1.75)
Oligosperm	35.67% (193/541)	2.30(1.69–3.13)	30.06%(95/316)	2.07(1.39–3.10)	0.093	1.29(0.96–1.74)
Asthenosperm	72.96% (305/418)	11.19(8.03–15.63)	51.06%(216/423)	5.03(3.46–7.31)	<0.001[d]	2.59(1.94–3.45)

[a] Odds Ratio

[b] P-Value for Compare before and after Gluthatione treatment

[c] Reference category

[d] Statistically Different

[14] reported the hamster sperm nuclei treated by Dithiothreitol (DTT) decondensed more rapidly when microinjected into hamster oocytes. This result in the hamster suggests that spermatozoa with a lower chromatin packaging quality should transform into pronuclei more readily and possibly more rapidly. However, glutathione (GSH), the major intracellular free thiol, has been implicated as playing a pivotal role in these events. On the other hand, treatment of mature hamster oocytes in vitro with a specific GSH oxidant, diamide, reversibly inhibits decondensation of microinjected hamster sperm nuclei, and systemic depletion of ovarian GSH with L-buthionine-S, Rsulfoximine (BSO), a specific inhibitor of GSH synthesis bocks decondensation of mouse sperm nuclei during subsequent in vitro fertilization [2]. Recently, the addition of reduced glutathione (GSH) to a fertilization medium was reported to increase the fertility of frozen/thawed sperm of various strains of mice [15, 16]. GSH is a biological antioxidant that can protect mammalian sperm against the loss of DNA integrity and motility through oxidative stress [15, 17, 18]. It has been proved that the susceptibility of sperm and seminal plasma to oxidative stress is significantly greater in idiopathic infertile men with mutation in glutathione transferase enzyme [19]. The basic function of GSH in mammalian semen is related to its interactions with other systems as a preventive mechanism against ROS. This scavenging function of GSH helps to counteract the effects of oxidative stress in sperm cells, which could result in lipid peroxidation of plasmalemma, loss of motility, leakage of intracellular enzymes and damage of the chromatin [20]. In this study asthenosperm samples, with problem in sperm motility, responded to the gluthatione treatment better than oligosperm samples. As it was mentioned before, it could be hypothesized that gluthatione causes more positive effect on sperm motility. Even though the precise mechanism of the positive effect of GSH on these discussed processes is still unclear, it could be suggested that the extracellular GSH prevents lipid peroxidation of cellular membranes of both gametes by removing excessive ROS in the insemination medium. [6] Interestingly, the GSH-stimulated fertilization can be associated with an increase in the number of free thiols in sperm membrane and can help the acrosome reaction and sperm penetration. Because GSH can reduce disulfide bonds (−S-S-) to produce two free thiols (−SH) [15, 21, 22], the observed effects on fertilization in our study might be due to GSH ability to protect against sperm membrane hardening, thereby increasing sperm penetration. These can explain more penetration in gluthatione treated samples in this study. In one study, reduced glutathione treatment has been used during sperm washing and insemination on the subsequent fertilization dynamics and development of IVM porcine oocytes [23]. Although they have seen some increase in sperm penetration and pronucleus formation after glutathione treatment, this was not significant [23]. This result could be in consent with our results since the effect of glutathione on our normal and oligo sperm groups was not significant, although we have seen some increase as well. This study [23] never showed whether the sperms were normal or not, meaning that if the work would have been done on astheno sperm samples, the results could have shown the same significance as ours. Moreover, final GSH concentration in that study (maximum 0.5 mM) was much lower than the concentration in our study (10 mM), which means that higher amounts of glutathione is needed to affect the astheno sperm samples.

In conclusion, the present results, coupled with our previous research on sperm PCC [11] showed that, this phenomenon can be one of the reasons of some idiopathic infertilities and fertilization failures. Moreover, addition of GSH ameliorated sperm quality and reduced the damage probably due to its strong antioxidant and reducing property. Treatment of sperms, especially asthenosperm samples, with GSH can be used in IVF or ICSI cycles to improve sperm quality and gain more success.

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