Study the effects of glutathione on fertility potential *in vitro* of male diabetic induced mice

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Abstract:

Background:

Diabetes has been found to induce various subtle molecular changes important for sperm quality and function: The mammalian spermatozoa are rich in polyun- saturated fatty acids, making them particularly sensitive to the deleterious effects of lipid peroxidation, which may result in irreversible loss of motility and a low level of fertilizing capacity. An antioxidant, glutathione (GSH) a polypeptide is important in biological oxidation-reduction reactions and improves sperm motility.

Objective:

The study was aimed to investigate the effects of GSH injection on healthy and diabetic male mice fertilization potential *in vitro* following *in vitro* fertilization (IVF)

Materials and Methods:

Inducing diabetes in mice through intra peritoneal alloxan injection. The treatment with GSH divided into two periods 10 and 20 days, studying the *in vitro* fertilization outcomes.

Results:

Glutathione daily IV injected in a period of 10 to 20 days for the diabetic induced male mice significantly increase the sperm concentration and motility and decrease the abnormal and dead sperms percentages in treated group compared to untreated group. The Fertilization rate was significantly ($P<0.05$)increased in mice treated with GSH compared to untreated one.

Conclusion:

Glutathione injections may decrease the oxidative stress caused by diabetes and enhance the *in vitro* fertilization potentials in male mice.

Keywords: Glutathione, diabetic mice, *in vitro* fertilization

Introduction

Diabetics have high levels of oxidative stress, which basically means too many free radicals and not enough antioxidants to neutralize them[(1)]. Diabetics also have low levels of intracellular glutathione. The high levels of oxidative stress and the low GSH levels further complicates the diabetic state which leads to even higher levels of oxidative stress and even lower levels of GSH[(2)]. Glutathione, being the master antioxidant, would naturally be the best choice as an antioxidant. Furthermore, inflammation leads to and contributes to insulin resistance. Glutathione, on top of being the most potent antioxidant, is also a powerful ant-inflammatory[(3)].

It has long been known that spermatozoa produce reactive oxygen species (ROS), mitochondria being the main source of the ROS production. Furthermore, leukocyte infiltration of semen is associated with decreased fertility due to leukocyte-produced ROS[(4)]. Thus oxidative stress is a major factor in the aetiology of male infertility. At the level of the isolated spermatozoon, ROS attack can induce lipid peroxidation and DNA fragmentation disrupting both the motility of these cells and their ability to support normal embryonic development. [(5)]. A large number of independent clinical studies have demonstrated a correlative relationship between male

infertility and evidence of oxidative stress in the ejaculate[6]. In the suppression of antioxidant enzyme expression, a concomitant increase in peroxidative damage, the disruption of spermatogenesis and an increase in germ cell apoptosis[7]. However, spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma or in spermatozoa itself to prevent oxidative damage. An antioxidant that reduces oxidative stress and improves sperm motility could be useful in the management of¬ male infertility[8]. Antioxidants are the agents, which break the oxidative chain reaction, thereby, reduce the oxidative stress[9].

In this study the results of IVF of ova by epididymal sperm obtained from diabetic induced mice treated with GSH will be elucidate.

Materials and methods

Experimental Animals

Male albino mice *(Mus musculus)* of age six months were kept in aluminum and plastic cages the dimensions of which were 260mm x 200mm x 140mm. They were supplied with mice food and water *ad libitum*. Mice were provided by ("Mario Negri" Institute for Pharmacological Research, Milano, Italy) and were bred at Charles River Italia (Calco, Lecco, Italy). They were housed at a temperature of 21 ± 1°C with relative humidity of 55 ± 10% and 12 hours light/dark cycle. Procedures involving animals and their care were conducted according international laws and policies[10]. The animals were grouped into four groups; each group consisted of six mice. Group 1: negative control: A group, Group 2: diabetic group + GSH: B10 and B20, Group 3: diabetic group: C10 and C20, Group 4: positive group + GSH: D10 and D20. Mice were sacrificed by cervical dislocation.

Diabetes mice induction[11]

Inductions of diabetes in mice were as follows:

1. A 48- Hour fasting.
2. IP Alloxan injection in (150) mg /kg body weight.
3. After 30 min. of injection the starvation period was ended.
4. After 10 days of injection the blood sugar level was checked by Accu check portable device.

Treatment with reduced glutathione[12]

The doses were prepared, depending on human doses(10 mg/ kg); therefore the mice dose account as 0.35 mg/ mice of normal antioxidant dose. The effective dose was counted as ten times dose used to get the 11.37 mg/mice in 0.5 normal saline 0.9 % daily for B and D group IV through the tail in two periods: 10 days and 20 days.

In vitro **fertilization (IVF) protocols**

Super ovulation program (SOP) and oocyte collection were done as described by Al- Dujaly and Al-Saadi[13]

1. The adult female mice (11 weeks) age were injected IP with 10 IU pregnant mare chorionic gonadotropin (PMCG)
2. After 48 hr. of the 1st. injection, were injected IP with (10 IU) human chorionic gonadotropin (hCG).
3. After (1416-) hrs. female mice were sacrificed and the whole reproductive system was carefully obtained in Petri-dish; thoroughly washed by normal saline 0.09 %.
4. Under a microscope, a slash was made in the ampulla with a fine needle and dragged out the ovulated oocytes to the medium as shown in figure (1).

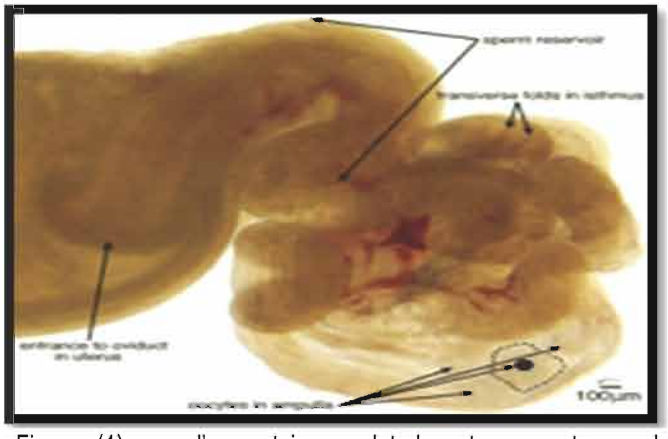

Figure (1) ampulla contains ovulated mature oocytes scale magnification 100x light microscope.

5. By using glass pasture pipette, the oocytes were collected to a new drop of cultured medium (RPMI-1640) + drop of HCG, incubated for (2- 4) hrs. at 37°C in Co2 5% humidity incubator *for in vitro* maturation (IVM) .

2. *In vitro* **fertilization (IVF) protocol and sperms collection**

1. Sperms from adult male mice were collected after sacrificed by cervical dislocation and getting the testis with the epididymus out the animal and washed thoroughly by normal saline 0.09 %.
2. The sperms were collected in the Earls salt medium drop. Sperm counts were made on epididymal sperm released from a single epididymis of each animal figure (2).

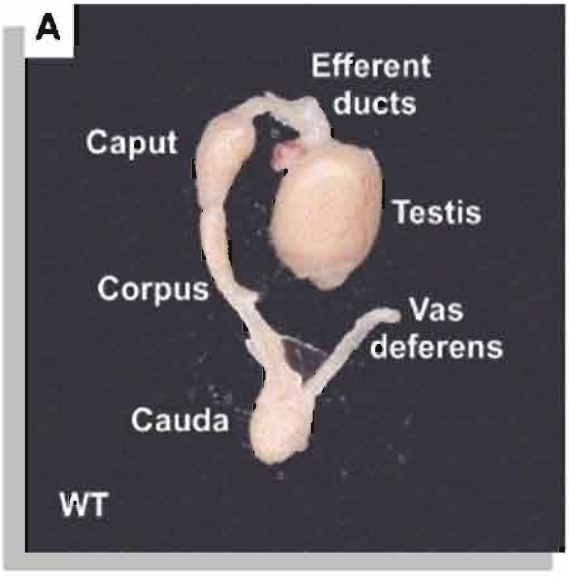

Figure (2) male mice reproductive apparatus,(14)

The tissue was minced with scissors, and then incubated in 0.5 ml of sperm motility buffer (135 mM NaCl, 5 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 30 mM HEPES pH 7.4, 10 mM sodium lactate, 1 mM sodium pyruvate, 20 mg/ ml bovine serum albumin, 25 mM NaHCO3). Sperms were allowed to swim up for 30 min at room temperature. Sperm motility was visually monitored under a phase-contrast microscope. Numbers of epididymal sperm were determined by hemocytometer counts either undiluted or diluted 10-fold.

All counts were made in duplicate and averaged, morphology for the sperm>s head and tail. Viability dead/live % (eosin red staining (sigma), were used). The temperature had to be controlled in range of 37◦C in normal humidified environment.

3. Assisted reproduction techniques according to (13): drop of warm 37◦C RPMI-1640 medium in a fertility dish +drop of 5 oocytes from IVM medium + 2

4. µl (250 x 105) of sperms preparation, moderate humidity.

5. Drops of paraffin oil (0.2 ml) were added around the fertility drop of medium to keep the fertility medium out of dryness.

6. The prepared dishes were incubated in 37◦C humidified CO2 5 % for overnight for fertilization.

Statistical Analysis

The data were statistically Analyzed by using analysis of variance (ANOVA) test to show the effect of different factors in the studied parameters. The least significant difference (LSD) was used to compare between means (15)

Results

Sperm evaluation and *in vitro* fertilization

The data in table (1) revealed that the sperm evaluation according to sperm concentration per one micro liter recorded the lowest significant value to the diabetic groups (B and C). The (B) group did not change significantly in comparison with the (A) control group.

The morphologically abnormal and dead sperms per one micro liter showed the significantly lowest value for (B20), the diabetic treated mice. (D10), which showed a significantly higher sperm concentration per one microlitre and (D20) with a significantly higher sperm motility in the culture medium. But, with group (C20), the 20 days of diabetes mice showed the significantly higher abnormal sperm morphology and dead per one micro liter percentage values recorded between all the groups.

Table (1): The sperm characters according to the experimental groups

Groups of mice	Sperm concentration m/ml	Abnormal morphology percentage	Dead sperms/1 µ percentage	Sperm motility percentage
A	1.833.333 ± 12.5	6.60 ± 0.09	18.3 ± 1.03	88.30 ± 4.25
B10	4.400 ± 0.45 a	15.00 ± 0.45	22.00 ± 1.56	87.50 ± 4.25
B20	15.813 ± 0.62	3.60 ± 0.07 a	4.66 ± 0.07 a	80.00 ± 3.79
C10	4.500 ± 0.76	6.50 ± 0.03	7.82 ± 0.26	68.33 ± 3.53
C20	8.462 ± 3.47	40.83± 2.13 b	30.86 ± 1.89 b	31.66 ± 1.73 a
D10	4.786.186 ± 42.69 b	7.23 ± 0.17	14.00 ± 0.78	81.66 ± 4.07
D20	3.436.185 ± 31.56	3.70 ± 0.07	7.90 ± 0.62	97.00 ± 4.91 b

Values: mean± SEM, significant level: P>0.05,
No. mice in each group =6 males
a: the lowest significant value, b: the highest significant value, A: control group, B10: diabetic induced mice treated with GSH for 10 days, B20: diabetic induced mice treated with GSH for 20 days, C10: diabetic induced mice after 10 days diabetes, C20: diabetic induced mice after 20 days of diabetes, D10: healthy mice treated 10 days with GSH, D20: healthy mice treated 20 days with GSH.

Figure (3) shows a images of vital(a and b) and abnormal sperms forms (c) with (d) which is a cross section of testis under an electron microscope showed the normal developing spermatozoon and (e) is the fully developed normal mice sperm under an electron microscope.

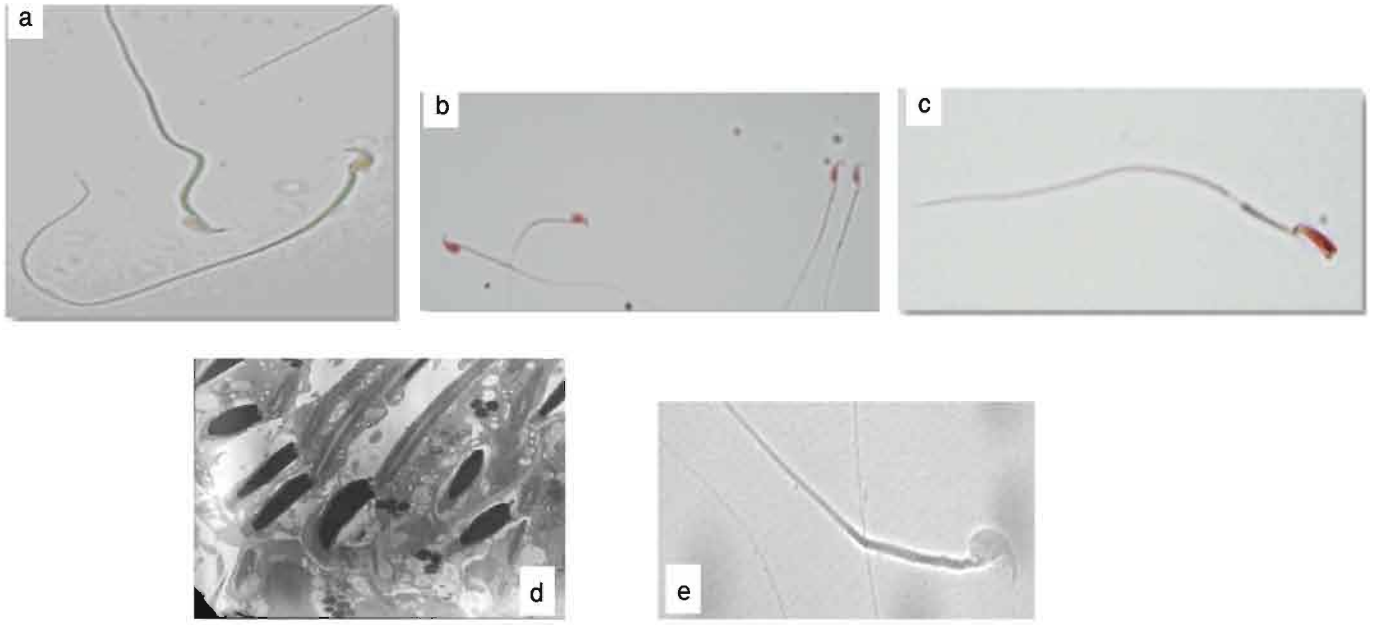

Figure (3) mice sperm, (a:a live sperms, b: dead sperm, c: morphologically abnormal sperms under light microscope power 40x (a: vital sperms, b: dead sperms, c: abnormal sperms) stained by eosin), (D: Sperms in testis cross section, e: normal mouse sperm (under electron microscope) scale bar 5 µm), (captured by the author).

Figure (4) (f) shows the healthy unfertilized oocyte surrounded by cumulus cells super ovulated from female mice 11 weeks of age collected from ampulla under light microscope 40X. Figure (4) (g) shows magnification of one of the ovulated oocytes. All the oocytes in this study have the same criteria, as shown in figure (4),

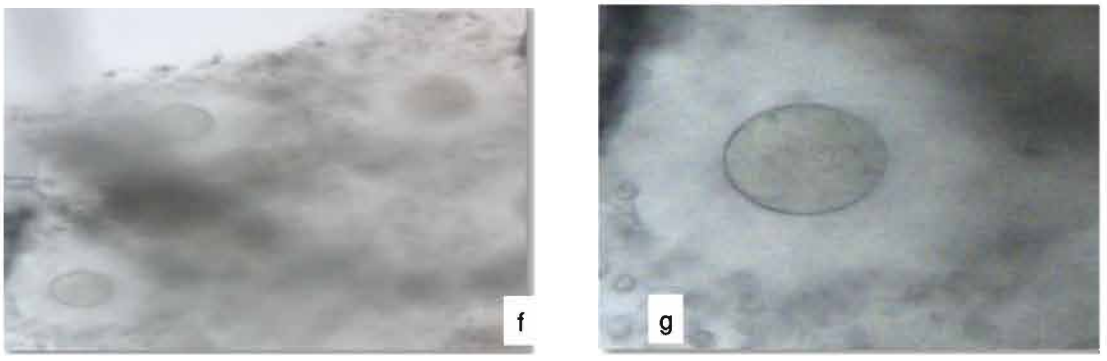

Figure (4) ovulated oocytes, F: ovulated mouse oocytes from ampula surrounded by cumulus cells under 40x power light microscope, g: mature mouse oocyte surrounded by cumulus cells under 40x power light microscope magnified in Microsoft office picture manager, (Captured by the author).

figure (5), which shows that (H): normal one day mouse embryo, (I): normal 2 days embryo (under 40x power dissecting microscope).

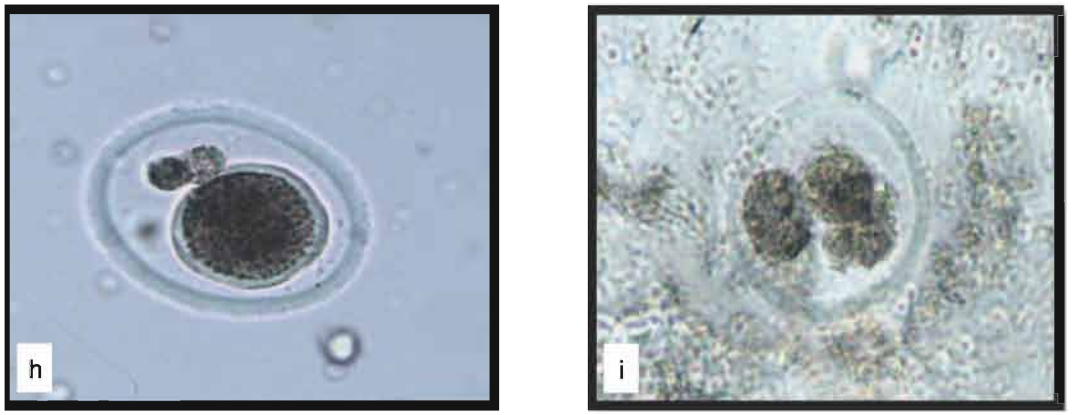

Figure (5) fertilized oocytes evaluation under dissecting microscope,H: normal one day mouse embryo, I: normal 2 days embryo (under 40x power), (Captured by the author).

According to these evaluations, table (2) recorded the percentages of the normal and abnormal fertilized oocytes. (Figwes

Table (2): The *in vitro* fertilization percentage evaluation differences according to the experimental groups:

Groups of mice	Fertilization percentage	Abnormal embryo development percentage
A	78.66 ± 3.67	0.00 ± 0.00 a
B10	38.00 ± 1.89	0.00 ± 0.00 a
B20	63.00 ± 2.65	0.00 ± 0.00 a
C10	16.83 ± 1.57a	70.30 ± 2.89 b
C20	14.70 ± 0.86 a	46.00 ± 1.74
D10	81.16 ± 3.58	0.00 ± 0.00 a
D20	83.30 ± 4.08 b	1.60 ± 0.64

Values are mean± SEM, significant level: P≤0.05,
No. mice in each group= 6 males,

A: control group, B10: diabetic induced mice treated with GSH for 10 days, B20: diabetic induced mice treated with GSH for 20 days, C10: diabetic induced mice after 10 days diabetes, C20: diabetic induced mice after 20 days of diabetes, D10: healthy mice treated 10 days with GSH, D20: healthy mice treated 20 days with GSH.

Discussion

The data in table (1) agreed with[16] and[17] in the highest significant sperm concentration for (D) group, the healthy treated group due to the effect of antioxidants on the testis performance in sperm production.

While the study by Desai, *et al.*,[18] showed no negative or positive effects on sperm concentration. And that disagree with the results which significantly affected by oxidative stress caused by diabetes, the significant affects on the sperm concentration of the (B) group than on the (C) group, which gives a basic role for the antioxidant GSH effect on enhancing the testis function producing more normal healthy viable sperms; even if it does not reach the control healthy level, it is significantly higher than the diabetic group. [19;20] in their study they pointed out that sperm motility is affected by the oxidative stress, and that agreed with the results recorded in table (1), This shows that the role of antioxidant GSH had not exceeded the normal standards in sperm movements but had therapeutic possibilities in diabetic males under an oxidative stress condition. The morphologically abnormal and dead sperms per one micro liter showed the significantly lowest value for (B20), the diabetic treated mice. This result agreed with a suggestion made by (6) that antioxidants protect sperms from oxidative stress and keep it vital. This suggestion agreed with (D_{10}), which showed a significantly higher sperm concentration per one microlitre and (D_{20}) with a significantly higher sperm motility in the culture medium. But, with group (C_{20}), the 20 days of diabetes mice showed the significantly higher abnormal sperm morphology and dead per one micro liter percentage values recorded between all the groups. The significantly lowest sperm movement, according to[21], shows that diabetes oxidative stress leads to an increase in the number of abnormal dead sperms. The negative correlation between the motility percentages and the dead or abnormal sperm percentages per one microlitre in a manner that the less movement the greater abnormal dead sperm percentages[22] agreed with this correlation.

Dead sperms lose the membrane selective permeability and let the stain come inside which turn sperms to pink under a light microscope while the a live sperms were not stained, so sperms look transparent or green under microscope. All the oocytes in this study have the same criteria, as shown in figure(4), to detect all the abnormality fertility outcomes that belong to sperm factors as suggested by[23]. The fertility results, with the sperm evaluation reveded strong evidence on *in vitro* fertilization outcomes, good sperm quality probably gives good *in vitro* fertilization outcomes[24]. It showed significant improvement for the percentage of increasing normal fertilization *in vitro* and decreasing abnormal embryo development. It has been Estimated that this could mean that lowering the ROS activity could protect the sperms morphology and viability, hence fertility performance.

The results of this study agree with a conclusion drawn by Vernet[26], who, assumed that GSH antioxidants led the normal healthy sperms to good fertility outcomes.

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