Pharmacological action and therapeutic effects of glutathione on hypokinetic spermatozoa for enzymatic-dependent pathologies and correlated genetic aspects

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Summary

In this study, the pharmacologic substance reduced glutathione (GSH) was used in men with hypofertility problems linked to varicocele, in bulls with spermatozoa hypomobility due to varicocele, and in rabbits with dispermy caused by cryptorchidism. An efficacious therapeutic effect of increased motility of the spermatozoa was seen in subjects who were submitted to appropriate doses of glutathione I.M. GSH also showed some neutralizing effect on the catabolytes produced during spontaneous or induced peroxidation processes of the unsaturated lipids contained in the membranes of male germinal cells. The genetic aspects of the involved enzymes were also evaluated and the research was extended *in vitro* by incubating samples of spermatozoa with arachidonic acid homogenates, L-tryptophan, hematein, and with an addition of glutathione. The results showed that polynsaturated fatty acid metabolic substances (PUFA) play an important role in the acrosomal reaction of spermatozoa and that GSH has a determining role in increasing the motility of spermatozoa with consequent improved fertilization. The spermatozoa of bulls provided us with a valid model to study for the morphostructural, biochemical and pharmacological analyses of human spermatozoa.

Key words: Glutathione; Spermatozoa; Free radicals; Mitochondria.

Introduction

Oxygen, which initially has the power of increasing the speed of spermatozoa progression, is successively very harmful due to its peroxidating power. A cascade of events takes place causing serious damage to the structure and functions of the male gamete. Because of this an increased degree of permeability ensues causing a partial loss of the constituents of the spermatozoa including some enzymes that are indispensable for the metabolism of glutathione. In fact, a deficit of GSH is responsible for, or at least plays an important part in, some pathologies of spermatozoa hypomotility.

In the past this spermatic hypokinesis was sometimes called idiopathic due to the ethiopathogenic difficulties. These processes are much more evident in cryopreserved spermatozoa than in fresh spermatozoa. Therefore, in our study samples of fresh spermatozoa and cryopreserved samples from the same ejaculation were analyzed.

There are multiple causes that have a negative effect on GSH, including those of genetic origin with pathological predisposition in which the defect can be ascribed to an enzyme deficiency. The correlated pathologies bring about an incapacity of the spermatic cells to maintain the necessary concentrations of glutathione thus causing an anti-oxidant/pro-oxidant imbalance. In these cases GSH can be used as support therapy to restore the physiologic constitution of polyunsaturated fatty acid (PUFA) in the membrane of spermatic cells. In fact, the substrate of the glutathione/peroxydase/reductase system represents an efficacious defense mechanism for the lipid perioxidation phenomena of the spermatozoa with an increase in fertilization power. This has been demonstrated by some researchers [1] who incubated human spermatozoa with arachidonic acid homogenates, L-tryptophan and hematein with an addition of glutathione. The same researchers showed that metabolic substances of some polyunsaturated fatty acids play an important role in the acrosomal reaction of spermatozoa and in fertilization.

Another important aspect is the lipid metabolism in spermatic cells for the production of energy, for the structure and also for the composition of the spermatic cellular membrane whose composition of phospholipids is rich in PUFA. During the maturation phase, the germinal cells and those that have reached the epidiymis are dependent on oxidized enzymatic systems that favor the occurrence of biochemical reactions. The cells rapidly combine with free radicals which are timely neutralized to prevent lipoperoxidation damage. These biochemical processes involve enzymes such as catalase, peroxidase glutathione types 1, 2, 3, 4, superoxide-desmutase 1, 2, 3 and reductase glutathione. However, any enzymatic imbalance caused by eventual oxidative stress can also have a genetic influence with pathological consequences on the male germinal cells [2]. Many of these enzymes use glutathione as a co-factor and are present in variable quantities in different processes of the evolutionary maturation of spermatozoa. In the seminal plasma glutathione starts biochemical reactions that utilize GSH, by combining with free radicals and because of the inter-

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vention of antioxidant enzymes as previously described. Thus, glutathione has an extremely fundamental role in the defense of the spermatic cell membrane against lipoperoxidation and from an increasing degree of PUFA unsaturation. The ratio of the reduced form of glutathione (GSH) and its oxidized form (GSSG) is a sensitive indicator of oxidative cellular stress. Experimental cell depletion of GSH, which is an index of strong oxidative damage, starts the mechanism of apoptosis with consequential cell death [3]. The state of glutathione is homeostatically controlled by a continuous balance between its synthesis (through GSH synthetase), its recycling of the oxidated form of GSSG (through GSH reductase) and its utilization by enzymes involved in oxidative stress and in the metabolism of the xenobiotics.

DNA mutations in the genes that code for enzymes involved in glutathione synthesis, like those in the genes that code for the enzymes involved in the recycled oxidated form (GSSG) to the reduced form of glutathione (GSH), bring about an enzymatic deficit. This lack causes cellular depletion of GSH with serious consequences regarding the damage caused by cellular oxidative stress. Recently nucleotic variations or polymorphisms (SNPs: single nucleotide polymorphisms) have been identified in the genes that code for some of the antioxidant cellular enzymes and utilize GSH as a co-factor. Other non-genetic causes of intracellular depletion of GSH are also possible.

It should also be kept in mind that abnormal thermal increases at the testicular level can alter intracellular enzymatic activities. In fact, if an enzyme is heated, it is denatured with consequent breaking of the polypeptide chain and loss of its enzymatic activity, thus demonstrating that the integrity of the primary structure of a protein has to remain such as to express its biological activity. These classic processes of testicular hyperthermia take place in some pathologies that are responsible for sterility, including varicocele and crytorchidism [4].

Our study was carried out using quantitative cytochemical doses in enzymatic activities connected with energy metabolism such as cytochrome oxidase and the lactate dehydrogenase. The investigations were performed during spontaneous lipoperoxidation (without inducers) or induced by Fe++ and ascorbate which markedly accelerates these processes. The study was also extended to the activity of the enzyme glucose-6-phosphate dehydrogenase, a key enzyme in the pentose phosphate pathway, which through the production of NADPH (nicotinamide adenin dinucleotide-phosphate-hydrogenates), controls the intracellular concentration of reduced glutathione. The substrate of the glutathione/peroxidase/reductase system represents an efficient defense mechanism against the phenomena of lipoperoxidation in spermatozoa with increased fertilizing power.

Pharmacological aspects and mechanism of action of glutathione

In this study we believed it was indispensable to evaluate, even if only as an overview, some biochemical aspects of GSH to determine its real pharmacological applications. Glutathione is largely diffused in nature being present in all animals, plants, and microorganisms. The largest concentration of GSH is found in the liver but it is also present in the spleen, kidneys, testicles, spermatides, and spermatozoa; the possible shortage of glutathione impairs the normal processes of reproduction. We are currently conducting a study on a group of herbivores (bulls and rams) with the aim of correlating hypofertility and alimentation without GSH.

Glutathione (γ-glutamil-cysteinil-glycine) is a tripeptide resulting from the union of glutamic acid, cysteine and glycine, and it plays a very important role in the protection of male germinal cells during oxidation processes. GSH controls the chemical and physical balance inside and outside the spermatic cell; it also protects the mitochondria of spermatic cells from the harmful effects of free radicals. In cases where the enzymatic equilibrium of the GSH cycle breaks, spermatozoa undergo significant damage including reduced motility and structural alterations that can compromise fertility.

The glutathione molecule can exist both in a reduced (GSH) or oxidated (GSSG) state. The oxidated form is a dimer in which two glutathione molecules are united by a disolphure bridge between two residues of cysteine. It plays an extremely important role in the protection of cells from oxidation processes that usually take place due to cellular aerobic metabolism. Additionally GSH protects cellular membranes including those in the mitochondria from the harmful effects of free radicals and from lipoperoxidation. The SH group in cysteine, present in the GSH molecule, is a powerful nucleophile which intercepts the electrophile substrates. In fact, GSH has a remarkable capacity to give electrons as a negative potential of the redox couple GSH/GSSH ($E'_0 = -0.23$ v). GSH is involved in a variety of metabolic processes such as detoxification of xenobiotics, the reduction of hydroperoxides, the synthesis of leukotriene and prostaglandins, the maintenance of the structure of membrane proteins and it is a co-factor of numerous anti-oxidant enzymes. The different functional capacities of the molecule are possible due to the chemical properties of the thiol group which can participate in oxi-reductions reactions, exchange of the thiol group reactions, formations of thioethers, and can act as a scavenger of free radicals. In fact, the importance of glutathione is widely demonstrated in the protection of spermatic cells from different reactive species of oxygen, from electrophylic composites deriving from metabolism of xenobiotics and from free-radicals. GSH also contributes in the recycling of other anti-oxidant substances such as vitamin E and possibly also carotinoids.

The state of glutathione is homeostatically controlled with a continuous balancing between the synthesis of GSH, its recycling from the oxidated form GSSG to GSH and its utilization by specific enzymes involved in oxidative stress and in the metabolism of xenobiotics (peroxidase, transferase, transhydrogenase, and transpeptidase).

The glutathione-peroxidase enzyme, with glutatione as a co-factor, plays an important antioxidating function at the intracellular level and disactivates the peroxide free radicals that are responsible for the breakage of cellular membranes. Free radicals cause, among other things, a negative impact on DNA and RNA. There are four classes of glutathione peroxidase. Class 1 is selenium dependent, composed of tetramer and its gene is located in 3p21.3. Class 2 is also selenium dependent and its gene is located in 14q24.1. Class 3 is an extracellular type mainly present in plasma and its gene is located in 5q32-q33.1. Class 4 is also called phospholipid hydroperoxide glutathione peroxidase (PHGPx = GPX4). It is also present at the level of the cellular membrane of the mitochondria of spermatides and, differently from the other forms which are tetrameric, it is monomeric and its gene is located in 19p13.3.

The imbalance or deficit of some enzymes involved in the cycle of glutathione brings about genetically detected pathologies. Enzymatic deficiencies can be determined hereditarily due to mutations in the genes that codify the involved enzymes. Moreover, some polymorphisms (SNPs, single nucleotide polymorphism) are nucleotidic variants present at least in 1% of the population and are due to the genes that code for antioxidant enzymes that use glutathione as a factor. All this can cause some functional variations with consequent reduction of the reducing power of the GSH/GSSG system.

The synthesis of glutathione is mediated by two enzymes; in fact, cysteine and glutamate are initially combined by the gamma-glutamil cysteinil synthetase enzyme, whose gene is located in 6p12. Subsequently gamma-glutamil cysteine reacts with glycine to form GSH which is a reaction catalyzed by GSH synthetase whose gene is located in 20q11.2.

After GSH has been oxidated to GSSG by antioxidant enzymatic reactions that utilize reduced glutathione (GSH) as a source of electrons, the recycling of GSSG to GSH is mediated by a reaction catalyzed by the glutathione reductase enzyme, of which a citosolic and mitochondrial form exist and whose gene is located in 8p21. This enzyme uses the NADPH co-enzyme as a source of electrons. NADPH, the main source of reducing power of GSH, comes mainly from the shunt of penthose phosphate by the action of the glucose-6-phosphate dehydrogenase (G6PDH) enzyme. The hereditary deficiency of this enzyme is transmitted with X-linked modality and involves the incapacity of the cell, including germinal cells, to maintain the necessary concentration of reduced glutathione with a consequent imbalance of the antioxidant/pro-oxidant system.

Glutathione and spermatic cells

Glutathione has proven to be an indispensable antioxidant for sperm since it has a positive effect on the motility of spermatozoa [5-7]. GSH together with selenium is an essential enzymatic co-factor of phospholipid hydroperoxide glutathione peroxidase (PHGPx). This enzyme is present in spermatides as a soluble peroxidase and it persists in mature spermatozoa as a cross-linked enzyme of the oxidation of insoluble proteins. In the midpiece of mature spermatozoa, the PHGPx protein represents at least 50% of the material contained in the capsule of the mitochondria [8]. In rats the genetic evidence of the sequenced cDNAs of GPX (epididymal glutathione peroxidase) has been demonstrated [9]. Other researchers have shown that, in experimental animals and in humans, the imbalance of the selenium contained in the capsules of mitochondria blocks the development of spermatozoa in the maturation phase since GPX modulates lipid peroxidization with consequent benefits for fertilization of spermatozoa [10]. PHGPx (selenoenzyme phospholipid hydroperoxide glutathione peroxidase) activity is expressed in the testicle only after puberty, disappears with hypophysectomy and is partially restored with gonadotrophic treatment [11]. Other researchers have shown that the MEP24 protein is a peroxidase-like molecule that acts with the GSH present in the epididimus head of mice [12].

Another concept to consider concerns the polyunsaturated and phospholipid fatty acids that are in the cellular membrane of spermatozoa which are extremely susceptible to damage that could be caused by oxidation. During the production of sperm, precise GSH regulatory processes occur which control the concentration of reactive oxygen, hydrogen peroxide, and nitric oxide. The disruption of this delicate balance causes, among other things, the formation of free radicals that directly damage spermatic cells and the deficit of glutathione should be considered as a possible etiology of male idiopathic sterility. A logical explanation for this is that glutathione, being the most powerful and important of the antioxidants produced by the organism, is the co-factor of the glutathione peroxidase enzymes. These enzymes have an antioxidant function at the intracellular level and disactivate the free radicals responsible for the rupture of cellular membranes. Free radicals also cause a negative impact on DNA and RNA. The remarkable disintoxicating capacity of GSH is related to the faculty of chelating, heavy and toxic metals such as lead, cadmium, mercury and aluminum; once chelation has occurred they are transported away and eliminated. It has been shown that, due to the lack or shortage of GSH in the epididimus of the testicle and in the liver, alchilating agents bring about cytotoxic effects in the already mature male germinal cells [13]. The explanation could be correlated to the cytotoxic effect of alchilating agents related to the alchilation of DNA components. These same agents have the property to undergo remarkably electrophilic biochemical reactions through the formation of cross-links with the target molecules. The above-mentioned reactions end up in the formation of covalent bonds (alchelation) with various nucleophilic substances including free radicals.

It should also be noted that, after their hydroxilation, the alchelating chemotherapeuticals become detoxificants by enzymes of the glutathione S-transferase group through the conjugation of molecules of reduced glu-

tathione (GSH). The glutathione S-transferases (GSTs) are a multigenetic family of enzymes present in all organisms. In humans they are especially found in the liver but also in the testicles and are codified by five classes of genes. There are also variants that have been associated to greater genotoxicity of alchilating substances, including that of male germinal cells, due to reduced activity of the detoxification system mediated by these enzymes together with the action of reduced glutathione.

The use of GSH has also been proposed for cases with oligozoospermia so as not to further aggravate any possible damage of the sperm caused by contact of healthy spermatic cells with pathological cells during *in vitro* manipulations in assisted reproduction [14]. It has also been shown that, during the very first phases of *in vitro* fertilization, glutathione brings about maturational improvement during the formation of the male pronucleus of the pig [15].

GSH therapy instead has not shown a significant increase in the quantity of seminal fluid or in the number of spermatozoa.

The mechanism of action of GSH therapy can also be studied because of its significant physiological presence in the seminal plasma. Various studies have in fact shown that glutathione after systemic administration increases its concentration in the biological fluids of seminal plasma where it can thus play its physiological and therapeutic role. Some researchers have performed rigorous controls by administering doses of GSH and, separately, equal quantities of placebo to people; the results showed that glutathione could be a valid therapeutic means in some specific andrologic pathologies related to hypokinesis of spermatozoa [16].

Materials and Methods

It is important to stress that bull spermatozoa provided a valid model for the study due to the similarities with human spermatozoa. The anatomical, functional and pharmacological similarities coincide perfectly in the human and bovine domains as we have widely documented in other studies [17].

As a main evaluation of the different spermatozoa exams, motility was considered above all using only physiological parameters higher than 20-25 μ/sec.

Experiment 1

This experiment served as a preliminary control study and different ejaculations from two bulls were used. One of the two, identified by the letter *A*, had already proved to be a reproducer due to its optimal fecundating power and was trained to ejaculate into an artificial vagina for the collection of spermatozoa which were cryopreserved and commercialized on a large scale. All spermatic parameters of this bull were controlled and resulted to be physiologically optimal. The other bull, identified by the letter *B*, was considered inadequate for reproduction because he was affected by bilateral varicocele and with scarce fecundating power and therefore was destined for butchering. The control exams we made on bull *B* showed marked spermatozoa hypokinesis. Moreover, different attempts of *in vitro* fertilization were carried out but the results were almost always negative.

Experiment 2

For this protocol we decided to use bull *B*, considered hypofertile. The bull was treated for 90 days with 6,000 mg (10 mg/kg) of glutathione I.M. per day. After this period it was made to ejaculate into an artificial vagina and the collected spermatozoa resulted to be normokinetic.

The study was extended *in vitro* with a second protocol using samples of hypokinetic spermatozoa from bull *B*, affected by varicocele. These spermatic cells, which were collected even before the treatment with GSH and which showed very low motility, were incubated at 38.5°C (temperature adequate for bovine gametes) for one hour with the Swim-Up method by Pellet in a TALP culture field with the addition of 1 mg/ml of GSH. The results showed that spermatozoa had acquired normokinesis. We proceeded with *in vitro* fertilization for further controls to check for the fertility of treated semen and the results were positive.

From the two protocols it can be seen that in pathologies with spermatic hypomotility due to varicocele the therapeutic effect of glutathione has an important role both *in vivo* and *in vitro*.

Experiment 3

Human spermatozoa from a young man who had to undergo surgical intervention for varicocele with grave spermatic hypokinesis were used. He was treated with 600 mg (10 mg/kg) of GSH I.M. for 60 days. After this therapy the results of the spermatic exams showed normokinesis of the spermatozoa.

Also in this case our research was extended *in vitro* to hypokinetic spermatozoa collected before the patient was treated with GSH I.M. Spermatozoa were divided into two distinct samples identifiable with the letters *A* and *B*; 1 mg/ml of GSH was added to only sample *A*. The two samples were incubated at 37°C (ideal temperature for human gametes) for one hour with the Swim-Up method by Pellet in Menezo B_2 medium. The results showed that only the spermatozoa identified with the letter *A* had clear motility. Instead hypokinesis persisted in spermatozoa not treated with GSH identified with the letter *B*.

With this experiment it has been unquestionably shown that GSH protects and at the same time enhances the motility of spermatozoa treated both *in vivo* and *in vitro*.

Experiment 4

In this experiment protocols were adapted from those already applied in a previous study [4]. Here samples of spermatozoa from four fertilized rabbits were used; everyone was easily identifiable by the letter *A, B, C, D*, respectively tattooed in the right earlobe. Preliminary exams showed that their spermatic cells were normokinetic. Three of the four rabbits (*A, B, C*) were submitted to bilateral cryptorchidism moving the testicles into the abdominal cavity and leaving them there for four days. The exam of the seminal fluid collected afterwards using an artificial vagina showed that at least 15% of spermatozoa were dead and almost all those remaining had hypokinesis with straight but very slow movements. These hypokinetic spermatozoa were then divided into three distinct samples identified by the letters *A, B, C* and 1 mg/ml of GSH was added to only the sample tagged with the letter *A*. The three samples were incubated simultaneously at 38.5°C (temperature adequate for rabbit gametes) for one hour with the Swim-Up method by Pellet in a Menezo B_2 medium. The results showed that the spermatozoa identified by the letter *A* had a clear motility with respect to those tagged with letters *B* and *C*. Therefore it has been shown

that GSH protects and at the same time enhances spermatozoa treated *in vitro.*

A second protocol was carried out *in vivo* on the same three rabbits (*A, B, C*) affected by astenospermia caused by induced cryptorchidism maintained for eight days (a double period with respect to the previous protocol). Afterwards, cryptorchidism was discontinued and 25 mg (l0 mg/kg/day) of GSH for 60 days were administered to the three animals. At the end of the therapy their spermatozoa resulted to be normokinetic. Thus once again the administration of glutathione demonstrated its therapeutic capacity in relation to male gametes.

A third protocol was carried out on the rabbit tagged with the letter *D*. This animal underwent cryptorchidism of the right testicle. After eight days the exams of the spermatozoa collected from the epididimus of the right testicle with induced cryptorchidism showed that the spermatozoa were hypokinetic. On the contrary the spermatic exams of the left testicle without cryptorchidism showed spermatic normokinesis. Afterwards, this same rabbit was treated with GSH and the subsequent spermatic exams performed on the semen of the right testicle retained for induction showed the recovery of normal motility of spermatic cells confirming the therapeutic effects of glutathione.

Experiment 5

Using the previous methods other protocols were carried out to control some of the enzymatic mechanisms involved in the cycle of glutathione in relation to the functional activity of spermatic cells [18, 19]. Both fresh and cryopreserved spermatozoa from three bulls of high geneology (Holstein Friesian) aged three to four years were used. The same germinal cells were used as a non-treated control (CTR) and collected at different times (from 0 to 180 min) during aerobic incubation at 37°C in PBS. It was taken into consideration that during incubation either spontaneous lipid peroxidization occurs or it occurs in PBS with the addition of sodium ascorbate (0.5 mM) and iron sulfite (0.05 mM) as promoters of lipid peroxidization (induced lipoperoxidation). Spermatozoa were then submitted to quantitative cytochemical reactions to demonstrate the cytochrome oxidase activities (COX) according to Frasch [20]; lactate dehydrogenase (LDH), according to Butcher [21] and glucose-6-phosphate dehydrogenase (G6PDH), according to Butcher and Van Noorden [22]. The microphotometric dosages, expressed in an arbitrary unit (a.u.) of integrated optical density (IOD), were performed with a Vickers M85a integrative microdensitometer at a wavelength of 480 nm for COX and of 585 nm for LDH and for G6PDH. The obtained data were submitted to statistical analysis of variance with the Scheffe test for multiple comparison and only the differences with $p \leq 0.01$ were considered significant.

Results, Discussion and Conclusions

The cytochrome oxidase activity resulted to be significantly decreased in bull spermatozoa; however, this decrement appeared to be more marked in cryopreserved spermatozoa with respect to fresh spermatozoa during the course of spontaneous lipid peroxidization .

The lactate dehydrogenase activity showed different trends in the two experimental conditions:

a) In the course of *spontaneous* lipid peroxidization such activity gradually and significantly decreased from the beginning to the end of incubation both for fresh and for cryopreserved spermatozoa, and such decrement resulted to be more marked for the cryopreserved spermatozoa;

b) During *induced* lipid peroxidization, the intensity of reaction for both fresh and cryopreserved spermatozoa first significantly decreased with respect to non-treated controls, and subsequently progressively and significantly increased in the IOD values followed by a gradual and significant decrease in the intensity of reaction. Both of these two last phases in the trend of values of IOD seemed to be early. The final decrement during incubation was more marked for cryopreserved than for fresh spermatozoa.

The activity of glucose-6-phosphate dehydrogenase in fresh incubated spermatozoa resulted to be somewhat detectable cytochemically for both the considered media. Starting after only 15 min of incubation, such activity increased gradually and persisted in the course of spontaneous lipid peroxidization for the entire period of incubation. Instead, during induced lipid peroxidization, after 90 min, the values of IOD significantly decreased. In cryopreserved spermatozoa such activity was not cytochemically detectable for the entire period of incubation.

Both for fresh and cryopreserved spermatozoa the significant decrement of cytochrome oxidase activity in each of the experimental conditions could have been due to inactivation of mitochondrial enzymes caused by products of lipid peroxidization. In fact, mitochrondria are particularly vulnerable with respect to lipid peroxidization because of the high content of unsaturated lipids and sulphidrilic groups. The data are also in agreement with the elimination of fructolytic and respiratory activity shown by spermatozoa of another species during lipid peroxidization [23]. As to interpreting the decrease in lactate dehydrogenase activity – observed in both in fresh and cryopreserved spermatozoa during the entire period of incubation in spontaneous lipid peroxidization and in the first period of induced lipid peroxidization – it may be hypothesized that there was some sort of inhibition of this activity. By analogy this is supported by some data in the literature describing a significant decrease of lactate oxidation in peroxidated ram spermatozoa [24].

The subsequent increase in the lactate dehydrogenase activity observed during induced lipid peroxidization could reflect the occurrence of membrane modifications which, correlated with an increased permeability of the membrane itself, would allow a greater access of exogenous NAD^+ co-enzyme in the locus of the reaction site.

Finally, the subsequent decrease in lactate dehydrogenase activity could be correlated with deeper modifications of the structural integrity of the membrane with a consequent loss of the enzyme itself. Thus, there is a pathological increment in the formation of free radicals of oxygen at the mitochondrial level. This is due to a change in the conditions of electron flow from donators of nicotinamide adenin-dinucleotide-hydrogenates (NADH) succinated to molecular oxygen, and in addition to a reduction in efficiency of the endocellular mecha-

nisms of anti-oxidating defense. This loss of control of electron flow may be seen as an actual "diversion" from the usual pathway. In other words, in these conditions a "flight" of electrons takes place outside the normal sequences of the transfer chain of electrons capable of firmly holding the partially reduced forms of oxygen at the level of its active sites up to their complete reduction to H_2O. Thus an increment occurs in the concentration of free radicals which starts the process of peroxidization of membrane lipids. Such event also involves a flight of electrons and the formation of free radicals of endocellular O_2, and thus a cascade of events takes place that produces significant damage to the structure of the spermatozoon. In fresh spermatozoa lipid peroxidization is an expression that, through the production of NADPH, controls the intracellular concentration of glutathione which is the essential substrate of one of the most important enzymatic defense systems with respect to the phenomena of lipid peroxidization. Lipid peroxidization takes place both spontaneously and when induced, and most likely causes the activation of the pentose phosphate pathway (glucose-6-phosphate dehydrogenase activity). This activity remains high and continuously increases gradually during spontaneous lipid peroxidization. The same activity decreases instead after the first 90 min of incubation during induced lipid peroxidization. Such decrement is probably due to inactivation of the enzyme itself which, as a SH-dependent enzyme, can interact with lipid peroxides. This last hypothesis could also justify the absence of such activity in cryopreserved spermatozoa where there is greater damage due to the radical and non-radical products of lipid peroxidization on the cellular physiology of the male gamate. This damage could cause the inactivation of glucose-6-phosphate dehydrogenase during the entire period of incubation both with and without the promoters of lipid peroxidization. Instead, by adding glutathione, a double anti-radical mechanism is primed: a trap action and buffer action. Essentially, GST competes in the transfer of electrons by subtracting those that are responsible for the excessive formation of auto-oxidable compounds of the chain itself from which the formation of O_2 and consequently of OH depend on. This "trapping" action allows the flight of electrons outside the sequences of the transportation chain to be limited and also for the unbalanced flow to be normalized. Thus, glutathione is a drug that prevents the formation of free radicals of O_2 and consequently hydroperoxides and lipid peroxides with a peculiar mechanism of drainage of drifting electrons (trapping effect) caused by mitochondria in the electron transfer systems. This effect occurs because the mitochondrial function is activated and thus acts as an interceptor of fleeing electrons. In addition to this action of preventing the pathological formation of free radicals and oxidating intermediates, there is another action that neutralizes radical and oxidating products attributable to the buffer property of glutathione. It also favors saving and therefore increasing the availability of anti-oxidant defense systems of spermatozoa. During this biochemical reaction free radicals are particularly damaging by manifesting their effects with deep membrane alterations of the main gametes and a consequent increase in permeability with loss of intracellular contents such as enzymes and essential substrates including some adenosinic and pyrimidinic nucleotides [25].

From the results it comes out that enzymatic activities appear to be deeply modified by the autooxidation phenomena, that the presence of inducers of lipid peroxidization anticipates the time and accentuates the alterations of some aspects of cellular metabolism of the spermatozoon, and that cryopreservation makes the male gamete increasingly more vulnerable with respect to the damage caused by oxidative stress probably also through the inhibition of some enzymatic defense systems.

The role of free radicals and mitochondrial misfunction in the alterations of electron transfer to O_2 during oxidative phosphorilization has also been pointed out.

Thus glutathione, by being the first example of a drug able not only to neutralize free radicals but also to prevent their formation by capturing fleeing electrons and subtracting them from the reactions taking place in the production of radicals, represents a new opportunity for intervention.

In our study we also tried to investigate the possible collateral and undesirable effects of GSH. In this respect we treated various bull ejaculations with glutathione and the spermatic material was cryopreserved in paillettes. With this semen 16 cows were inseminated and nothing pathological was registered during the entire period of gestation. No anomaly was shown in calves before or after birth. In conclusion, the results have clearly shown that glutathione had no embryotoxic, fetotoxic, mutagenic or teratogenic effects.

The results of the clinical and pharmacological investigations of glutathione confirmed the validity of the theoretical assumptions on which the action of the drug is founded.

To conclude, in our experiments it has been possible to show that the pharmacological action of glutathione may be considered an efficient antiperoxidant in the damaging processes of spontaneous and induced lipid peroxidization in male germinal cells which, when treated with GSH, demonstrated an improvement in fertility.

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