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Changes in ATP level and iron-induced ultra-weak photon emission in bull spermatozoa, caused by membrane peroxidation during thermal stress*

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ATP level, cell motility and viability, oxygen uptake, pyruvate kinase activity, and ultra-weak photon emission (UPE) induced by red-ox Fe^{2+}-ascorbate cycling system were studied in fresh, in previously equilibrated in a glycerol diluent, and in cryopreserved bull spermatozoa, exposed to thermal stress by incubation of the cells at 44°C.

A sharp drop in motility and viability of fresh spermatozoa and even more so, of equilibrated and cryopreserved cells was accompanied by accumulation of ATP. When cell movement was totally inhibited, ATP utilization was decreased, while chemical energy continued to be produced by cell pyruvate kinase, one of the key glycolytic enzymes, which in spermatozoa is very active (6500 IU/g protein) and insensitive to feed-back inhibition by excess of ATP and L-cysteine.

Accumulation of ATP during incubation at 44°C in 0.9% NaCl was accompanied by a rapid decrease in oxygen consumption by fresh spermatozoa and an increase in Fe^{2+}-ascorbate induced UPE, followed by a sharp decrease in ATP level observed at the end of induced UPE measurement. The increase in photon emission due to lipid peroxidation was highly correlated with the increase in cell ATP level caused by thermal stress.

Bull spermatozoa represent an interesting experimental model. The cells, both fresh and equilibrated in a glycerol diluent, as well as those cryopreserved in liquid nitrogen and thawed, at the optimum temperature of 38°C sustain their motility and viability for more

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Abbreviation: UPE, ultra-weak photon emission.

than ten hours. During cell incubation at 44°C (6°C higher than the optimum temperature) thermal stress shortens the cell motility and viability time to a few hours only [1]. In such conditions the cells may undergo an auto-oxidative injury connected with formation of free radicals [2] which are normally involved in increased turnover of cellular components.

Reactive oxygen species are induced by the Fe^{2+}-ascorbate redox system which causes hydrogen peroxide accumulation.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$$

$$O_2^{\bullet-} + O_2^{\bullet-} + 2\,H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Since spermatozoa have little or no catalase activity [3], hydrogen peroxide formed in the presence of Fe^{2+} reacts with superoxide anion radical yielding hydroxyl radical.

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\bullet}$$

Hydroxyl radicals have especially harmful effect, leading to further "oxygen damage" of spermatozoa contractile proteins, and irreversible peroxidation of cell membrane polyunsaturated fatty acids [2, 3]. In this iron-induced system not only an increase in malonyldialdehyde formation was observed [3–5], but also an ultra-weak photon emission (UPE), which gives information about the overall biological effect of a stress on the cell.

Little is known about concomitant changes in UPE and the level of ATP as chemical energy carrier. ATP necessary for cell movement, formed by respiration and glycolysis, may also participate in photon emission. Therefore comparative studies of fresh, cryopreserved and equilibrated bull spermatozoa were undertaken to evaluate changes in cell ATP level, occurring during incubation at 44°C, especially before and after iron-induced UPE.

MATERIALS AND METHODS

Bull sperm from the Artificial Insemination Centre in Zabierzów (Poland) was collected into an artificial vagina (3–4 ejaculates) and divided into three portions of semen, free of seminal plasma. One portion contained fresh semen. Two other portions were equilibrated in commonly used egg-yolk citrate diluent (80 ml, 2.9% sodium citrate × 2 H_2O, 1.25 g fructose, 20 ml hen egg-yolk, 100000 IU penicillin, 0.1 g streptomycin) for 20 min at room temperature. After a period of 3.5 h in a refrigerator at 2–4°C, an additional amount of egg-yolk citrate diluent containing 10% glycerol was added (to make 3.5% final glycerol concentration), mixed and equilibrated for another 15 min. One portion was assigned for a direct study, the other one was cryopreserved, i.e. frozen into pellets on dry ice (–79°C) and transferred to liquid nitrogen (–196°C).

The studies were performed on fresh spermatozoa, equilibrated cells, and cells cryopreserved and thawed. The samples were centrifuged for 10 min at 2000 r.p.m. (400 × *g*), washed twice and re-suspended in 0.9% NaCl to obtain 60–100 × 10^6 cells/ml. In this way cell suspensions free of seminal plasma and of components of the egg-yolk citrate-glycerol diluent, were used [6]. The first measurement was made at "zero" time, the others ones after incubation in a water bath at 44°C for various periods of time.

The motility of the spermatozoa was determined microscopically and expressed as percentage of motile cells (moving in any direction and at any speed). Experimental error of measurement was ±6% [6].

The viability of the spermatozoa was assessed by supravital staining with specific membrane stains, eosin and nigrosin, according to Blom [7].

For detection and registration of UPE, the single photon counting method was used, employing a cooled (250 K) EMI 95580 B photo-multiplier tube, sensitive in the spectral range of 200–800 nm, as described previously [1, 6, 8–10]. Each measurement cycle started by determination of the background emission. Cell suspension in the cuvette was flushed with 99.5% oxygen at a flow rate of 5 l/h. Photon emission was induced with the Fe^{2+}-ascorbate system [6], at 0.22 mM ascorbate and 45 µM $FeSO_4$, final concentrations [4].

ATP concentration was measured in incubated cells spectrophotometrically by the enzymatic method of Adam [11], and in the extracts obtained by homogenization of cell pellets with 0.6 M perchloric acid, followed by centrifugation and neutralization with saturated $KHCO_3$ in the presence of methyl red.

Oxygen consumption was measured using a Clark's electrode (YSI 5300 Biological Oxygen Monitor) [12].

Pyruvate kinase (EC 2.7.1.40) activity in cell cytosols was measured by the spectrophotometric method of Bücher & Pfleiderer [13] using a pseudo-zero-order kinetic modification [14]. For preparation of cytosol fractions, the cell pellets were homogenized in a Potter-Elvehjem glass homogenizer, with 20 mM Tris/HCl buffer, pH 7.4, containing 115 mM KCl, 10 mM $MgCl_2$, and 2 mM EDTA. The homogenates obtained were centrifuged for 10 min at $20000 \times g$ at 4°C. Enzyme measurements, before and after 24 h dialysis in a refrigerator against the homogenization buffer, were performed in the presence of 5 mM 2-phosphoenolpyruvate as substrate. The reaction was started by adding 1.25 mM ADP (final concentration) in the absence or presence of ATP or L-cysteine (0.1 mM final concentrations). The results were expressed in IU/g protein.

Protein was determined by the method of Lowry *et al.* [15].

The reagents for ATP and pyruvate kinase measurements were obtained from Boehringer Mannheim.

RESULTS

The highest initial motility and viability were found in fresh spermatozoa (60%) and the smallest in cryopreserved cells (32–35%). During incubation at 44°C in 0.9% NaCl, the motility of fresh cells decreased, reaching zero value after 4 h. In cryopreserved and thawed cells, after 2 h of incubation, when 40% of fresh cells were still motile, the movements were seen only in about 10% of the cells. At that time the motility of equilibrated cells decreased from 40% to zero value (Fig. 1).

During incubation at 44°C, cell viability persisted for about 3 h at an almost unchanged level in fresh and equilibrated cells, and then began to decrease. The same stabi-

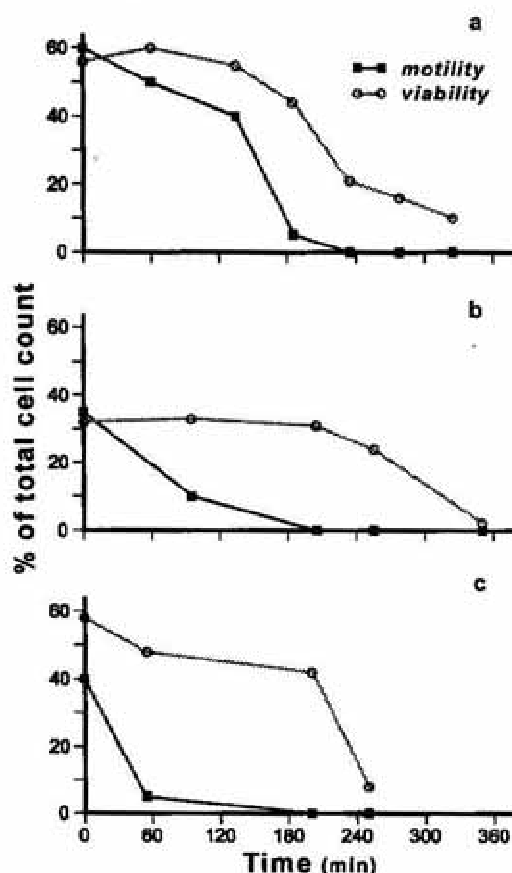

Figure 1. Bull spermatozoa motility and viability during incubation at 44°C in physiological saline (per cent of total cell count).

Comparison of cells: fresh (a), after cryopreservation (b), after equilibration (c).

lization of viability for 3 h but at a lower level was observed in cryopreserved cells (Fig. 1).

A sharp drop in motility and, to a lesser extent, the reduction in cell viability could point to inhibition of energy metabolism. In agreement with this assumption in fresh spermatozoa the oxygen uptake was found to decrease during incubation, parallelly with the disappearance of movement activity (Fig. 2). However, since the level of ATP in fresh spermatozoa increased during incubation at 44°C, to values three to four times as high as the initial levels, the suggestion of lack of chemical energy reserves for contractile proteins was rejected.

As ATP was accumulated, despite a decrease in oxygen consumption, it was sup-

posed that ATP formed in glycolysis was not utilized by contractile proteins.

Pyruvate kinase, the key glycolytic enzyme, directly involved in ATP formation had a very high activity in fresh spermatozoa supernatants: 6540 ± 1280 IU/g protein (n = 6). After dialysis against the homogenization buffer, the enzyme activity increased by 19.9% from 7470 to 8960. This was a result of removal of various low molecular effectors, e.g. sodium ions. The activity of such dialyzed enzyme was not changed after addition of an excess of exogenous ATP or L-cysteine.

Since the decrease in cell motility and viability cannot be explained by the lack of energy, another possibility is a damage of cell membrane integrity under the influence of free radicals. This can be seen during thermal stress as an increase in stain permeability, and as iron-induced ultra-weak photon emission (UPE) [1, 6, 8–10].

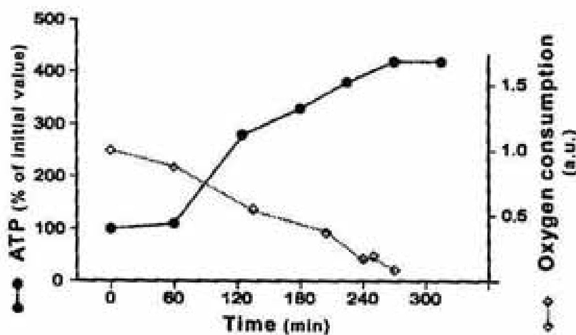

Figure 2. Oxygen consumption and ATP level during incubation of fresh bull spermatozoa at 44°C in physiological saline.

Oxygen uptake is expressed in arbitary units (a.u.).

The results of iron-induced UPE are presented in Fig. 3. After 3 h incubation at 44°C, a threefold increase in photon emission, in relation to the initial value (212 counts/10^6 cells), was found in fresh spermatozoa, followed by a slight downward tendency. The increase in photon emission intensity from cryopreserved spermatozoa, which were less motile and less viable, was only doubled (141 counts/10^6 cells) in relation to the initial value. The smallest increase by about 50% (117 counts/10^6 cells) in photon emission in relation to the initial value was observed in equilibrated cells (Fig. 3).

In all three types of the material studied, the increase in photon emission during incubation was accompanied by an increase in cell ATP level. Initial ATP levels in cells exposed to thermal stress were similar (1.16–1.52 nmol/10^6 cells after sample concentration in centrifuge). During incubation at 44°C, when motility disappeared and cell viability decreased, the ATP level increased in comparison to the initial value accepted as 100%, fourfold in fresh cells after 4–5 h, threefold in cryopreserved cells reaching a plateau after 6 h, and only twofold in equilibrated cells (Fig. 3).

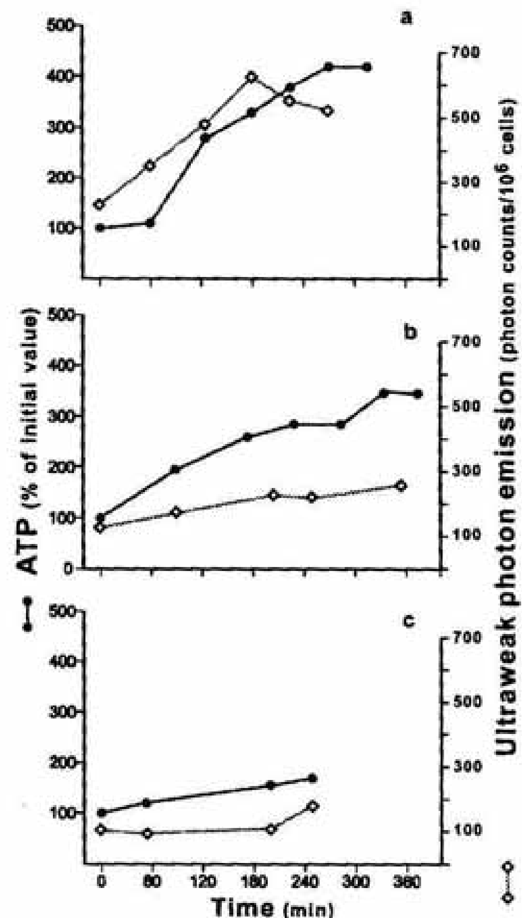

Figure 3. Comparison of ultra-weak photon emission (UPE) (photon counts/10^6 cells) and changes in bull spermatozoa ATP level (% of initial value).

Denotations as in Fig. 1.

In fresh bull spermatozoa, with the ATP level of 14.5 ± 2.8 nmol /mg protein (n = 4), after 3 h incubation at 44°C, when a threefold increase in ATP level was seen (324% of initial control), addition of the Fe^2 -ascorbate

cycling system induced photon emission, and caused after 30 min of chemiluminescence measurement a sharp decrease in cell ATP level to about 67% of initial control value.

DISCUSSION

At an early stage of studies on semen, Mann [16, 17] and other authors [18, 19] pointed to a close relationship between sperm energy metabolism and motility. Mann even suggested that the measurement of ATP content could provide an objective method for estimation of semen viability. According to Söderquist & Larsson [20], the ATP content was highly correlated with the progressive motility of sperm cells in fresh and cryopreserved semen, and the decrease in the ATP content caused a loss of motility. However, spermatozoa retained their motility even when the respiratory activity was abolished by cyanide or by replacing air with nitrogen [16]. Glucose (or fructose) was found to be indispensable if the spermatozoa were to remain alive under anaerobic conditions, indicating that the metabolism of bull spermatozoa is predominantly of a glycolytic character [16, 21].

According to Cascieri *et al.* [22], ATP level of bull spermatozoa corresponds to 0.26–0.44 nmol/10^6 cells (approx. 13–22 nmol/mg protein). However, much smaller values (0.15–0.35 nmol/10^6 cells, or 0.08–0.12 nmol/10^6 cells) [20, 21, 23, 24] were also reported. Therefore the influence of ATP estimation methods, as well as of procedures of ATP extraction or conditions of spermatozoa preparation, on the ATP level require further more systematic comparative evaluation, especially as ATPase activity in non-deproteinized semen may also diminish the results.

In our experiments ATP was accumulated, despite several hours incubation at 44°C in physiological saline without addition of exogenous substrates; this was presumably due to a previous irreversible loss of the motility. Gorus & Pipeleers [25] suggested that glycolytic metabolism of spermatozoa could be maintained for about 4 h at the expense of endogenously accumulated substrates and observed that after that time, in the absence of exogenous D-glucose it decreased in comparison to the control group without glucose. The presented results indicate that the sharp decrease in cell motility and cell viability cannot be explained by energy deficiency but, on the contrary, limitation of cell motility at 44°C corresponds to an even increased cell ATP level.

The decrease in oxygen consumption by fresh spermatozoa, i.e. inhibition of cell respiration during incubation at 44°C, could be a result of increasing auto-oxidative damage to mitochondria caused by thermal stress. However, in the absence of ADP and P_i as indispensable intermediates for oxidative phosphorylation [26], and in the presence of an excess of ATP, electron transport is inhibited (acceptor control), as is also the oxygen uptake [27].

In such a situation glycolysis is the only pathway of ATP formation. Normally, aerobically formed ATP is an inhibitor of glycolysis (Pasteur effect) [28] but in some cells this effect does not appear [29]. Lardy *et al.* [30] demonstrated that ejaculated mature spermatozoa did not show any Pasteur effect, and their aerobic and anaerobic glycolysis was similar.

Pyruvate kinase, one of the key, regulatory enzymes of glycolysis [31], which shows high activity both in fresh and cryopreserved spermatozoa supernatants, is directly involved in cell ATP formation. Among various pyruvate kinase isoenzymes [32], an over-expressed tumor variant of M_2 isoenzyme, which has no sensitivity to feedback inhibition by ATP [33–36], acquires sensitivity to inhibition by L-cysteine [33–35]. Over-expressed pyruvate kinase from bull spermatozoa is inhibited neither by an excess of ATP, nor by L-cysteine. In such a situation, ATP accumulated by pyruvate kinase can inhibit oxygen consumption by mitochondria [27] but not aerobic glycolysis. Thus the decreased cell motility and viability due to thermal stress seems rather to be a result of cell membrane damage [3–5] by free radicals.

Cells with damaged membranes do not show any spontaneous luminescence. But reactive oxygen species induced by the Fe^{2+}-ascorbate redox cycling system lead to further increase of "oxygen damage" to proteins and

lipids [4] which may manifest itself as induced UPE. This iron-induced effect can be considered as evoked by the previously initiated highly spermicidal lipid peroxidation [3–5], followed by disturbances in motility and membrane permeability.

It seems that the sequence of changes taking place under the influence of thermal stress is as follows: cell membrane damage, mobility and viability decrease, ATP accumulation, inhibition of oxygen consumption, induced increase in electron leakage, and stimulation of UPE, followed by lowering of the ATP level.

It is important to realize that spermatozoa are normally protected against free radicals by sperm plasma which contains a variety of antioxidants, e.g. ascorbic acid (0.14 mg/ml), as free radical scavengers preventing irreversible lipid peroxidation [4]. According to Mann & Lutwak-Mann [21] these substances play a beneficial role in sperm survival. Therefore spermatozoa washed and suspended in 0.9% NaCl are much more sensitive to lipid peroxidation [3].

In contrast to fresh cells, attention should be paid to only a slight increase in ATP level and UPE during incubation of cells previously equilibrated in a solution rich in fructose and fatty acids (e.g. from lecithin hydrolysis), saturating the cells and capable of cell membrane protection. The weak photon emission from equilibrated bull spermatozoa can be thus explained as due to the protective action of egg-yolk coat on cell membrane during incubation [5].

In preliminary studies a significant decrease in ATP level was observed after 30 min of iron-induced UPE measurement in comparison to control exposed only to thermal stress. A similar ATP decrease was observed by Mann *et al.* [3, 4] after iron-induced malonyldialdehyde liberation. The observed ATP decrease in a non-enzymatic reaction [37] could be rather a consequence of an increase in iron-induced lethal injury not only to cell membranes, but also to cytosolic proteins which became unable to take part in further ATP formation.

The existence of UPE proves that in certain conditions of exergonic lipid peroxidation or free radical recombination, living systems generate much more energy ($\Delta H \geq 400$ kJ/mol) at the short wave limit of the spectrum than in enzymatic reactions or the classical reaction of ATP hydrolysis to ADP. Such high enthalpy values indicate an "energetic disaster" in the cell, probably caused by electron leakage from the respiratory chain [38].

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