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ULTRA-WEAK LUMINESCENCE OF SPERMATOZOA

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Spontaneous and stress-induced ultra-weak luminescence (UL) of bull, ram and boar spermatozoa cells have been investigated using the single photon couting method and cut-off optical filters. Simultaneously, the motility and vitality of the cells were determined. The absolute value of intensity was evaluated to be of the order of 10^{-4}-10^{-5} photons/s per cell and for the first time correlated with motility and vitality. Environmental stress factors such as the osmotic pressure, strong illumination with white light, temperature, prooxidant substances and hyperoxic conditions enhance the intensity and light sum of UL with concomitant decrease of motility and vitality of cells. The iron-induced UL under oxic conditions covers a broad spectral range from 300 to 850 nm with the main emission band in the red part of the spectrum (λ>600 nm). The results indicate that UL is associated with the peroxidation of lipids in the membrane of spermatozoa cells which is a primary target of a majority of stress factors.

INTRODUCTION

Spontaneous ultraweak luminescence (UL) emitted from living organisms at the rate (intensity, I) of about $1\text{-}10^4$ photons/(s·cm^2) in the spectral region λ=200-400 nm is the manifestation of the radiative deactivation of electronically excited states. These excited states of molecules are endogenously generated in exergonic metabolic or metabolism-coupled side reactions occurring mainly within biological membranes (for review see Adam & Cilento, 1982; Cadenas, 1984; Slawińska & Slawiński, 1983, 1985; Jeżowska-Trzebiatowska, Kochel, Slawiński & Stręk, 1987; Jeżowska-Trzebiatowska, Kochel, Slawiński & Stręk, 1990; Popp, Li & Gu, 1992). Parameters of UL: the intensity I, kinetics $I=I(t)$, spectral $I=I(\lambda)$ and probability (photocount) $p(n,\Delta t)$ distributions depend on the character, energetics and rate of metabolic reactions. These parameters may reflect the physiological state and capacity of homeostasis of living organisms. Therefore, variations of these parameters elicited by external and internal stimuli may serve as a holistic, cumulative (integrative) measure of the response of an organism to the stimuli. Thus, analysis of the parameters provides information about changes in the rate, energetics of metabolism and physiological state of a tested organism or cell population (Schamhart & Van Wijk, 1987; Van Wijk & Schamhart, 1988; Van Wijk & Van Aken, 1991, 1992; Kochel, 1990, 1992; Slawiński, Ezzahir, Godlewski, Kwiecińska, Rajfur, Sitko & Wierzuchowska, 1992).

The majority of research on UL was performed with vegetative cells (Van Dyke & Castranova, 1988). Very little is known, however, about UL accompanying the activities of generative cells (Foerder, Klebanoff & Shapiro, 1978; Nakano, 1989; Takahashi, Totsune-Nakano, Nakano, Mashico, Suzuki, Ohma & Inaba, 1989). The natural chemiluminescence associated with fertilization of sea urchin eggs involves excited species: $O_2^{\bullet-}$ and cation radical of tyrosine (Takahashi *et al.*, 1989). Ho and coworkers (Ho, Ross & Saunders, 1992) reported that populations of synchronously-developing *Drosophila* embryos emit light after oviposition (when development begins). Still less is known about the UL of spermatozoa; the results of the first research on this subject had

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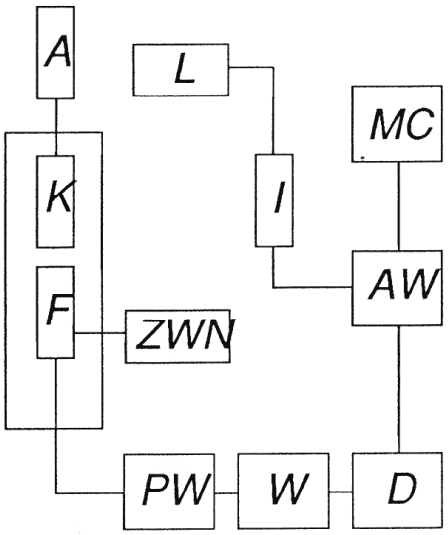


Fig. 1. Schematic presentation of the experimental setup for measurements of ultraweak spontaneous and photo-induced luminescence of spermatozoa by the single photon counting method. A — irradiation cuvette, K — measuring chamber, F — photomultiplier tube, PW preamplifier, W — amplifier, ZWN — high-voltage power supply, D — discriminator of pulse amplitude, AW — multiscaler, MC — microcomputer, I — triggering circuit, L — halogen lamp with filters.

barely appeared by 1992. Ezzahir and coworkers (Ezzahir, Godlewski, Kwiecińska, Sitko, Slawiński, Szczęśniak-Fabiańczyk & Laszczka, 1992a) reported on a photo-induced UL from bull spermatozoa, while Ezzahir *et al.* (1992b) and Erokhin *et al.* (1992) on the iron induced UL from ram and bull spermatozoa. It was found that these cells reveal extremely low-level spontaneous UL in unperturbated, physiological conditions. However, when stressed by detrimental environmental factors, such as strong illumination, osmotic shock, pro-oxidants and high temperature, they produce a distinct signal of UL (Slawiński *et al.* 1992; Laszczka, Ezzahir, Godlewski, Kwiecińska, Rajfur, Sitko, Slawiński, Szczęśniak-Fabiańczyk & Wierzuchowska, 1993, 1994). Therefore, in the present study we have investigated UL from suspensions of bull, ram and boar spermatozoa illumination, osmotic pressure, pro-oxidants and temperature. The results of these studies provide evidence of the (1) oxidative character of processes underlying UL and their localization within membranes, and (2) of a correlation between certain parameters of UL and physiological indices characterizing the status of spermatozoa influenced by some external factors.

MATERIAL AND METHODS

Preparation of cell samples

Fresh bull, ram and boar semen, collected at the Artificial Insemination Station in Zabierzów and the National Institute of Animal Production in Balice near Kraków, were evaluated for volume, concentration and motility. Our previous experiments have shown that correct measurements of UL of spermatozoa cells require cell suspensions free of the seminal plasma as well as commonly used egg diluters, for example: yolk-citrate diluter for bull (Ezzahir *et al.*, 1992a), milk-yolk diluter for ram and Kiev-formula for boar spermatozoa. Spermatozoa were separated from these fluids by two-fold centrifugation (400g, 10 min.) and resuspended in 0.9% NaCl (bull spermatozoa), Goetze phosphate solution (ram spermatozoa) or in 5% glucose (boar spermatozoa). In some cases, 2.9% trisodium citrate or water were used. The final concentration of cells was about $(1.44^{+}\text{-}0.10)\cdot 10^{8}$ cells/ml. Chemicals of analytical grade were obtained from POCH (Gliwice, Poland). Bi-distilled water was produced using all-glass apparatus.

Evaluation of physiological indices of the cells

The motility of the spermatozoa was determined microscopically and expressed as the percentage of motile sperm moving in any direction at any speed. The experimental error of determinations was ±5%. The vitality of the cells was assessed by supravital staining with eosin and nigrosin (so-called "life-dead" test introduced by Blom, 1950). This differential test is based on the capacity of a membrane's receptors to bind specific stains and bears a maximum experimental error ±15%.

Measurement of ultra-weak luminescence

For the detection and registration of UL, the single photon countig method (SPC) was used, employing a cooled (250 K) EMI 9558 QB photomultiplier tube sensitive in the spectral range 200-800 nm. The measuring equipment is shown in Fig. 1. and described in detail elsewhere (Ezzahir *et al.* 1992a, Rajfur, 1994). A glass cuvette containing 4 ml of cell suspension or medium was placed in the darkened sample compartment at a small distance from the photomultiplier tube. The temperature of the sample compartment was kept at an appropriate level maintained within ±0.1 K. Prior to the commencement of measurement, the samples were kept in complete darkness for 5 min. Each measurement cycle started by determination of the background emission (BG) resulting from the dark current of pho-

Tab. 1. Kinetic parameters of iron-induced chemiluminescence from bull spermatozoa cells ($2 \cdot 10^8$ cells/ml) incubated in various media at 43°C.

Medium	I_{max} [CPS]	dI/dt [cps/s]	τ_{max} [s]
2.9% citrate	420	9.6 ±2.1	81.0 ± 5
0.9% NaCl	450	6.2 ±1.35	105.0 ± 5
water	1900	212.0 ±30	19.4 ± 1

dI/dt — maximum relative rate of chemiluminescence increase
τ_{max} — time in which maximum emission intensity is reached

tomultiplier and long lasting luminescence from cuvette and shutter. Then the shutter was opened and the number of counts per 1 or 10 s recorded each 1 or 10 s interval and stored. Each sample was measured at least three times, the recorded data being expressed as a mean ±SD. To obtain hyperoxic cell culture, the cuvette containing cell suspension was flushed with 99.5% oxygen at a flow rate of 5 l per hour.

The irradiation procedure

For the study of a white-light effect, 4 ml of cell suspension was sucked into a syringe attached to the cuvette and irradiated 60 s with the white light (380-800 nm, 10^4 lx) from a 150 W tungsten halogen lamp. The light passed through a heat reflection filter which cuts off wavelenghts above 800 nm and below 380 nm. After irradiation, the sample was injected back into the cuvette; the dead time between the end of irradiation and the start of counting was 300±70 ms.

Iron (Fe^{2+})-induced chemiluminescence and lipid peroxidation

Induction of peroxidation of phospholipids and unsaturated fatty acids of the spermatozoa cell membranes was performed with the nonenzymatic Fe^{2+}-ascorbate system. Final concentrations of $FeSO_4$ and ascorbate were 50 µm and 0.5 mM (Laskowska-Klita & Szymańska, 1989). The process of lipid peroxidation was studied by the iron-induced chemiluminescence: injection of 0.5 mM $FeSO_4$ solution (final concentration) into the cell suspension resulted in the radical-mediated decomposition of lipid peroxides and hydroperoxides and concomitant light emission (chemiluminescence). Kinetic parameters of this emission such as the induction period, peak-height, light sum (integrated intensity) and slopes of the intensity-time curve $I=f(t)$ provided information about the rate and degree of peroxidation (Ezzahir *et al.*, 1992b).

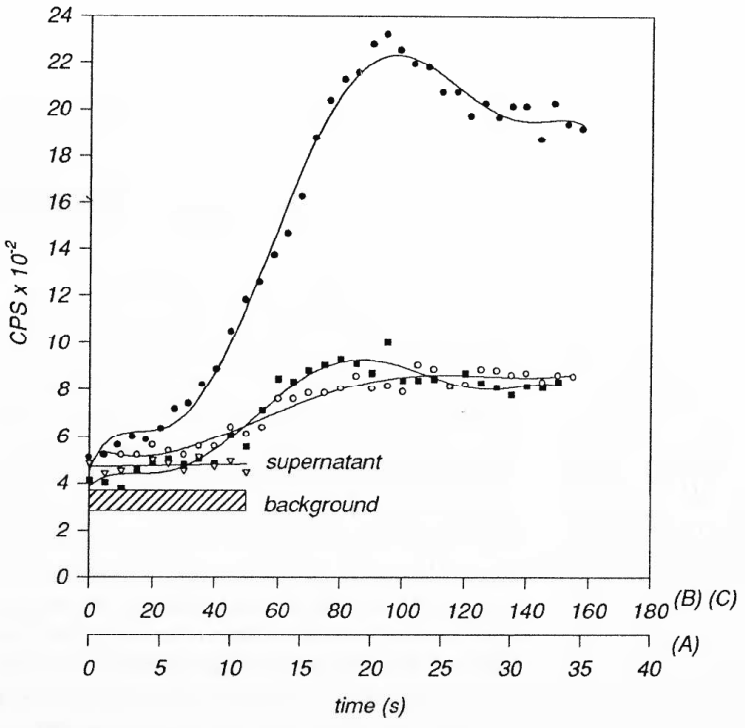


Fig. 2. The effect of osmotic stress on the iron-induced ultraweak luminescence of bull spermatozoa. Cells incubated in: A —water, B — 2.9% trisodium citrate, C — 0.9% NaCl. Addition of 500 µM $FeSO_4$ is indicated by the arrow.

Estimation of the spectral distribution of UL

The spectral distribution of UL from spermatozoa was analysed by interposing glass absorption-cut-off filters between the cuvette and the cuvette and the photocathode. Filters were inserted in a light-tight disc. Estimation of

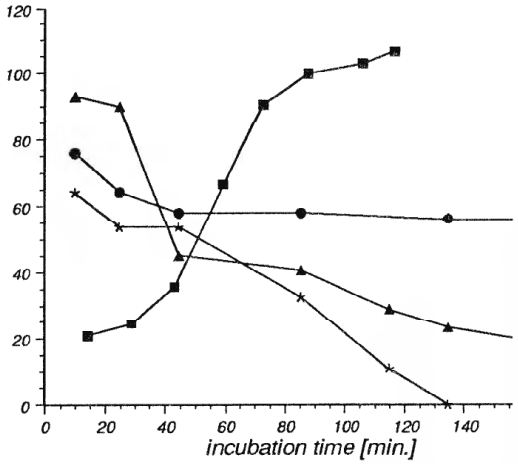


Fig. 3. The influence of incubation time at 43°C on the motility (%, stars), vitality (%, circles), induction time of I_{max} (min., triangles) and maximum intensity of luminescence (arbitrary units, a.u., squares) of the Fe-induced chemiluminescence of bull sperm cells.

the spectral distribution of UL was done according to Vassilev (1965).

RESULTS

A suspension of intact (unperturbed) bull, ram or boar spermatozoa emits extremely low spontaneous (intrinsic) UL, the intensity of which (signal, S) is close to background level (N) of the photon counting system. The value S/N≅1, and does not change in a statistically significant way during incubation up to 12 h at T<313 K. However, significant changes in the intensity I and kinetics $I=f(t)$ are observed when the cells are exposed to detrimental environmental influences. In the following chapters, the effects of osmotic, chemical, temperature or light stress are described.

Tab. 2. The influence of CH_2O on thawed spermatozoa ($6 \cdot 10^7$ cells/ml) in Y-Citr-NaCl

[CH_2O] %	Initial/Final % vitality	motility	Photo emission cps
0 (control)	68.3±3	50±7	95 ±2.69 (BG)
0.0005	55/29	50/5	2 ±0.18
0.001	55/30	50/2	4 ±0.18
0.0125	65/32	50/0	7 ±0.34
0.04	56/27	35/0	12 ±0.45
0.10	55/29	35/0	16 ±0.52
1.0	54/2	35/0	23 ±0.62

Osmotic stress

In this series of experiments, bull spermatozoa were resuspended in 0.9% NaCl, 2.9% citrate and distilled water. After different periods of incubation at 43°C, 4 ml of the cell suspension was injected into the cuvette and 1 ml of $FeSO_4$ (final concentration 500 μM) was added to decompose accumulated lipid peroxides and associated chemiluminescence. Data presented in Fig. 2 show that the addition of Fe^{2+} to spermatozoa incubated in water enhance is the I of chemiluminescence by almost 300% in contrast to spermatozoa incubated in a physiological solution. Kinetic parameters given in Table 1 indicate that the rate of chemiluminescence increase (dI/dt) is much higher, while the τ_{max} (time after addition of Fe^{2+} in which the maximum intensity is reached) is much shorter for spermatozoa subjected to the osmotic stress than for the cells incubated in a physiological solution. The dI/dt parameter that

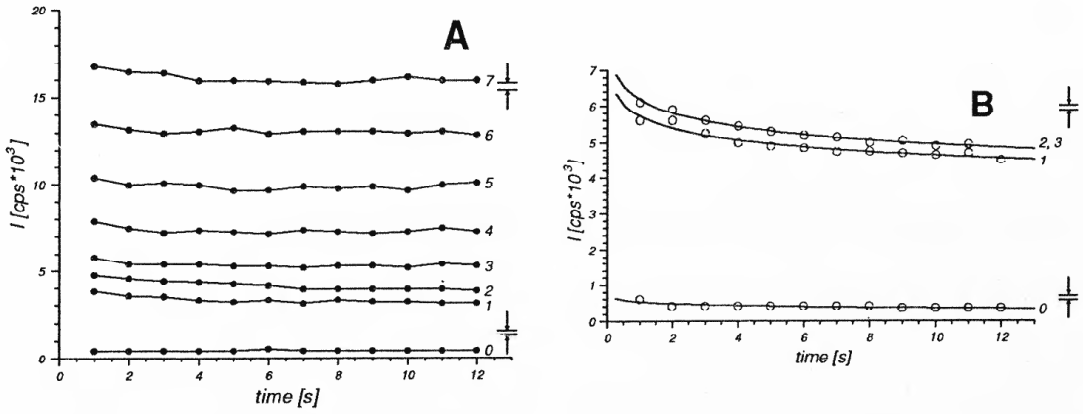


Fig. 4. The intensity of delayed photo-induced luminescence of bull spermatozoa cells ($1.4 \cdot 10^8$ cells/ml) successively irradiated with white light ($3 \pm 1.5) \cdot 10^4$ lx in: A — 2.9% sodium citrate; B — 2.9% sodium citrate + 5.7% formaldehyde; 0 — spontaneous emission of non-irradiated cells. 1, 2, 3... 7 — correspond to successive irradiation; irradiation time was equal to 60 s, measurements started 61 s after the end of irradiation. Horizontal dashes and vertical arrows at the right side indicate maximum error of photo-count.

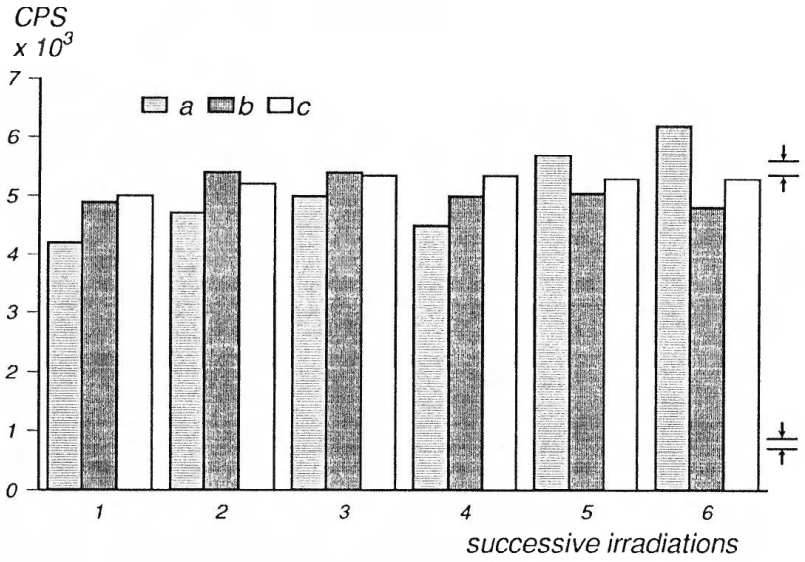


Fig. 5. The effect of successive exposure to white light on delayed ultraweak luminescence of bull spermatozoa cells incubated in: a — water; b — water + 5.7% formaldehyde; c — 2.9% sodium citrate + 5.7% formaldehyde (final concentration). Other conditions and symbols as in Fig. 4.

reflects the slope of the ascending part of the kinetic curve $I=f(t)$, i.e. the rate of the reaction which produces electronic excited states, appears to be the most sensitive parameter. Fig. 3 shows a correlation between I_{max} and induction period of chemiluminescence and biological activity of spermatozoa cells — motility and ability to bind eosin ("vitality"). The increase in incubation time was found to enhance chemiluminescence. In contrast to this, the "vitality" and motility decreased.

This finding supports the hypothesis that motility and "vitality" are affected by free radical formation and concomitant lipid peroxidation initiated by environmental osmotic stress.

Light stress

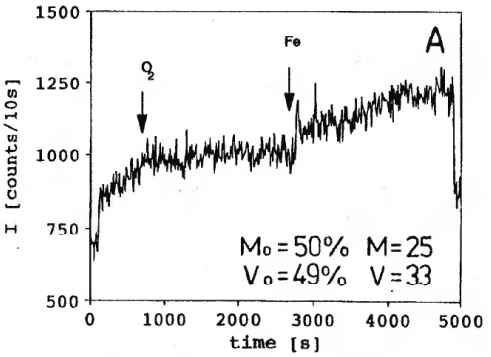
Only a few reports deal with the effect of light on the quality and physiological properties of mammalian semen (Roussel, Patrick, Kelgren & Guidry, 1964; Lubart, Friedmann, Levinshal, Lavie & Breitbar, 1992). The results of our experiments on the effect of white light on the UL and motility of bull spermatozoa are presented in Fig. 4 and 5. Irradiation of cells with strong light results in delayed UL, consisting of a fast (<1 s) and slow (1-50 s) component. The intensity of this photo-induced UL exceeds, by more than 10 times, that of spontaneous emission (Fig. 4).

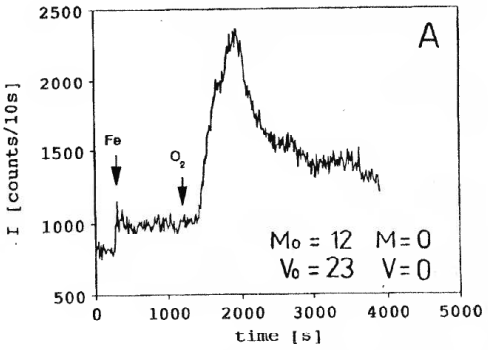
Photo-induced delayed UL is dependent on the vitality of spermatozoa cells: cells incubated in physiological solutions (0.9% NaCl, 2.9% trisodium citrate) are sensitive to light, whereas cells incubated in detrimental environments: water or formaldehyde solution, are not. Data presented in Fig. 4A indicate that successive periods of irradiation of intact spermatozoa with white light cause an increase in the intensity of delayed UL. After seven irradiation cycles, the enhancement of the intensity amounted to about 400%. Fig. 5 shows the effect of 6 successive irradiations on the I of delayed UL of spermatozoa cells incubated in three harmful solutions. In this case, no enhancement of the delayed UL is observed. The motility of cells is drastically reduced in a decreasing order: formaldehyde > osmotic stress (water) > light.

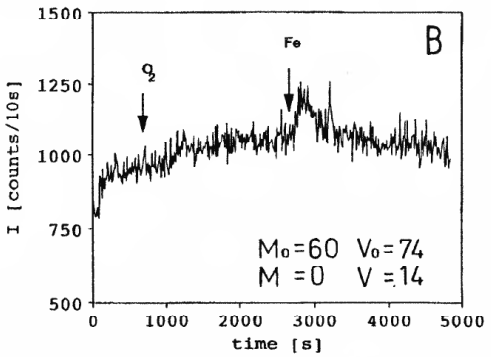
Thus, the enhancement of photo-induced delayed UL during irradiations is somehow linked with the viability of cells, probably by coupling photoprocesses to enzymatic reactions which take place in the living organisms. Possible interpretations of this effect are discussed below.

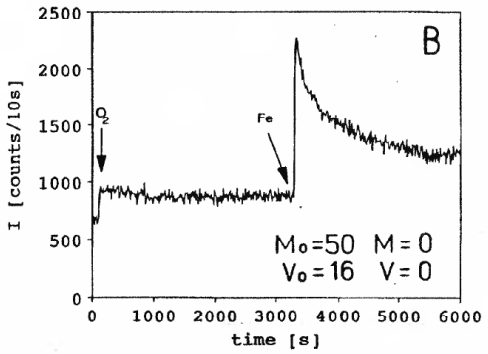
Chemical stress

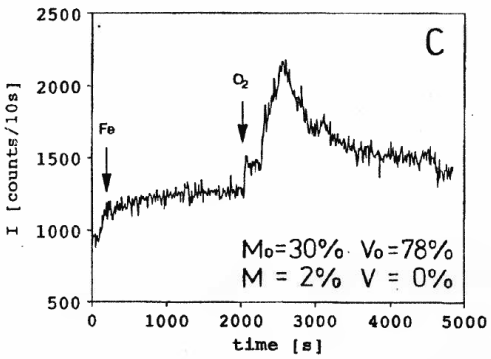
The effect of formaldehyde on UL. Thawed bull spermatozoa cells suspended in the yolk-citrate-NaCl diluter were treated with increasing concentrations of formaldehyde. Changes in motility M, vitality V and intensity I of spontaneous UL were determined for each concentration of formaldehyde. The corresponding data are given in Table 2. The most striking effect is a great difference in the response of the motility and UL to the same increments of formaldehyde. This chemical is a well known protein-denaturing agent and a strong depression of motility is, *a priori*, expected. However, the increase in the intensity of UL caused by the same changes in concen-











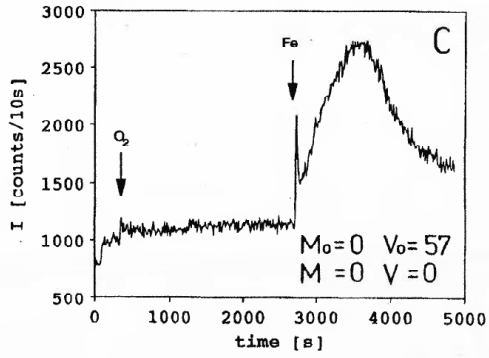


Fig. 6. Kinetics of ultraweak luminescence $I=I(t)$ of the suspension of spermatozoa cells from: A — bull, B — ram and C — boar (incubation time < 6h) before incubation. The final concentration of added $FeSO_4$ (arrow) was 25 μM, the rate of oxygen flow 5 l/h, temperature 40°C. M_0/V_0 and M/V —motility and vitality (%) before and after the experiment, respectively.

Fig. 7. Kinetics of ultraweak luminescence $I=I(t)$ from the suspension of spermatozoa from: A — bull, B —ram and C — boar after 12 h of incubation. Other conditions as in Fig. 6.

tration of formaldehyde is very weak. In order to quantitatively characterize the response of cells to a given concentration of formaldehyde, one can introduce the coefficient of sensitivity:

$$X = \Delta S/\Delta C$$
$$\Delta S = (S_{i+1} - S_i)/S_i)$$
$$\Delta C = (C_{i+1} - C_i)/C_i ,$$

where S_i are two consecutive values of M, V or I and C_i corresponding consecutive concentration of formaldehyde. From Table 2 it can be seen that X values change in a nonlinear way in parallel to the stressor concentration, the motility being the most sensitive and nonlinear. It shows a threshold-like behaviour in contrast to the vitality V that is gradually modified as concentrations of formaldehyde increase. The average value of X for UL is equal to 0.005 which means that spermatozoa cells do not change distinctly the level of spontaneous emission in response to formaldehyde.

The effect of pro-oxidants. The membrane of mammalian spermatozoa are susceptible to damage by oxygen as a consequence of lipid peroxidation (Jones & Mann, 1973; Erokhin *et al.*, 1992; Ezzahir *et al.* 1992b). If UL of spermatozoa is associated with lipid peroxidation within membranes, then the intensity and kinetics of UL should be sensitive to prooxidants. Therefore in our experiments pure oxygen (instead of air), added iron ions, and the iron-ascorbate system promoting lipid peroxidation were used. The effects of oxygen atmosphere, addition of Fe-ions and incubation time are presented in Fig. 6. and 7. As can be seen from Fig. 6, the air-equilibrated suspensions of spermatozoa cells exhibits very low UL intensity with the S/N ratio 1.20, 1.15 and 1.30 for bull, ram and boar, respectivelly. The addition of 25 μM $FeSO_4$ increases the S/N ratio to 1.50, 1.53 and 1.78, respectively. Then the exchange of an air atmosphere for O_2 results in a distinct increase of UL in the form of a peak that reaches the S/N ration of 3.00, 4.20 and 3.00, respectively in its maximum. In another series of experiments, the sequence of addition of pro-oxidants was the opposite. When O_2 was supplied first to the suspension of bull, ram and boar spermatozoa, the S/N ratio was 1.67, 1.83 and 1.17. The addition of $FeSO_4$ enhanced the S/N ratio up to the value of 2.08, 2.90 and 3.10. Comparison of this effect suggests that the redox couple Fe^{2+}/Fe^{3+} is a more efficient pro-oxidant than molecular oxygen alone in its ground triplet state $^3\Sigma_g^-$. Indeed, iron ions are known to promote radical chain reactions stimulating peroxidation of lipids in membranes of spermatozoa cells. Initial and final values of motility and vitality (before and after experiments) given in Fig. 6 and 7 indicate that the incubation of cells in the pro-oxidative environment drastically decreases their motility and vitality. These findings agree with the well-established detrimental effect of lipid peroxidation induced by iron compounds, oxygen and peroxides on spermatozoa (Jones & Mann, 1973; Strzezek *et al.*, 1992). Non-enzymatically induced peroxidation was performed by incubation of spermatozoa cells in the presence of 50 μM $FeSO_4$ + 0.5 mM ascorbate. Under such conditions, a significant enhancement of UL was observed. The S/N ration was increased to about 6 without significant change of kinetics depending on the concentration of reactants and temperature. All these data pro-

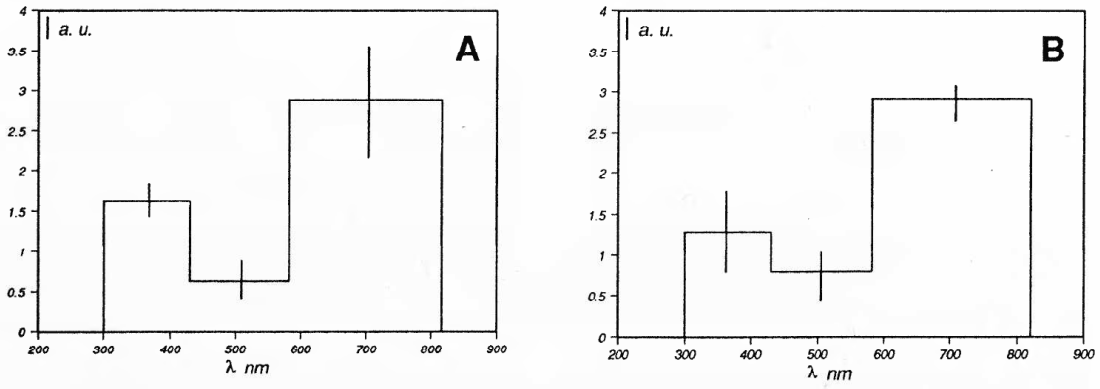


Fig. 8. Spectral distribution of the iron-induced ultraweak luminescence of spermatozoa cells ($2.5 \cdot 10^8$ cells/ml) — A, and liposomes of an egg yolk lecithin — B, incubated at 43°C. Concentration of lecithin is 10 mg/ml. Spectra were measured 300 safter maximum intensity of the Fe-induced emission was reached and calculated with the correction for the transmittance of cut-off filters, spectral sensitivity of the photomultiplier tube and decay of luminescence kinetic.

vide evidence that spontaneous UL of spermatozoa is associated with peroxidation reactions.

Spectral distribution of luminescence

Using the cut-off filters method — the technique most adequate for spectral analysis of extremely low light intenstities, we have attempted to evaluate the spectrum of UL from spermatozoa cells. However, the *S/N* ratio has appeared to be too low for reliable analysis in the case of spontaneous UL from cells under physiologically most favorable conditions. Therefore, we have measured the series of Fe^{2+}-induced chemiluminescence kinetics $I=f(t)$ for several cut-off filters giving the highest *S/N* values and $\Delta S/N$ differences essential for a statistically significant spectral analysis. The coarse spectral distribution of Fe^{2+}-induced UL from bull spermatozoa cells is presented in Fig. 8A. It can be seen that the emission from peroxidized cells covers a broad spectral range from 300 to 825 nm and the red band (600-825 nm) contributes most significantly to the total emission. The contribution of this band increases with the duration of the Fe^{2+}-stimulated peroxidation reaction, reaching the value of about 70% at 300 s after τ_{max} and then remains almost constant. This value does not significantly change with the incubation time of the cell suspension either. If the observed emission (Fig. 8A) originates from exergonic lipid peroxidation reactions in membranes, then its spectral distribution should be the same as that from a phospholipid model system. Therefore, we have prepared liposomes from yolk phospholipids and measured the emission spectrum from liposomes (Fig. 8B) using the same procedure as for spermatozoa cells. It can be seen that the spectrum observed during Fe^{2+}-induced peroxidation of phospholipids is the same within the limits of statistical error as that observed in the case of cells. Thus, the coincidence between the spectrum of the spermatozoa cells and a model system reinforces our prediction that the observed emission is associated with lipid peroxidation in cell membrane.

The effect of temperature and incubation time

Incubation of bull spematozoa cells at physiological temperatures 27°C and 36°C gave no statistically significant differences in the intensity of spontaneous UL. However, after extending the incubation time over 12 h, a distinct tendency towards an increase of UL was found. This tendency is clearly seen in Figs. 6 and 7. The effect of temperature was more distinct for the Fe-induced chemiluminescence at temperature exceeding the physiological range. Incubation of spermatozoa in 0.9% NaCl at 36° and 46.4°C during 6-12 h resulted in an approximately 2-fold increase of *I*. In this temperature range, the Van't Hoff coefficient $Q_{10}=I_{maxT+10}/I_{maxT}$ is about 2.0. Such a value of Q_{10} can be formally classified as characteristic of physical (Q_{10}=1-1.3) and enzymatic processes ($Q_{10} \leq 2$).

DISCUSSION

First reports on UL of spermatozoa cells were published in 1992 (Erokhin *et al.*, Ezzahir *et al.* 1992a, b) and until now the nature and source of this phenomenon, and its analytic-diagnostic potential are still poorly understood. The data presented in this work provide evidence that these highly specialized and important cells reveal extremely weak UL, under most favorable physiological conditions, the characteristics of which can be correlated with biological activity and peroxidative damage to cell membrane. Like mammalian and plant cells, the spermatozoa so far studied increase their UL emission when perturbed by detrimental stress factors. Thus, the osmotic stress (hypotonic conditions) imposed on bull spermatozoa by incubation in water, enhances the Fe^{2+}-induced chemi-luminescence (Fig. 2 and Table 1). The plasma membrane of sperm appears to have a limited area (Noiles, Mazur, Watson, Kleinhaus & Crister, 1993), which would result in membrane leakage or lysis, should a cell be placed in a medium sufficiently hypotonic to bring about swelling beyond the maximum volume. The mean critical tonicity at which the membrane disruption of bull spermatozoa occurs is 36 mOsm (Watson, Kunze, Cramer & Hammerstedt, 1992). Therefore, the cells incubated in water undergo progressive spermolysis and membrane disruption. The increased permeability of the damaged sperm membranes results in the leakage of intracellular enzymes into the extracellular medium and, under aerobic conditions, in uncontrolled peroxidation. Therefore, the addition of heavy metal ions with variable valency $M^{n\pm1}$ such as e.g. $Fe^{2+/3+}$ initiates the decomposition of hyperoxides and peroxides accumulated during the peroxidation:

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO^{\bullet} + OH^{-}$$
$$Fe^{3+} + ROOH \rightarrow Fe^{2+} + ROO^{\bullet} + H^{+}$$

$$\text{Sum. } ROOH \rightarrow RO^{\bullet} + ROO^{\bullet} + H_2O .$$

The chemiluminescense-promoting role of Fe-ions may be explained by the formation of hydroperoxides and the generation of radicals:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -}$$
$$O_2^{\bullet -} + H^{+} \rightarrow HO_2^{\bullet} \xrightarrow{e^{-}.H^{+}} H_2O_2 \xrightarrow{e^{-}.H^{+}} H_2O + HO^{\bullet}$$

Free radicals are known to initiate peroxidation of poly-unsaturated fatty acids in biological membranes. In these chain reactions, electronically-excited states are formed and then deactivated which manifests itself as an emission of light (UL). The dismutation of lipid peroxy radicals can be a source of either excited (*) carbonyls or singlet molecular oxygen:

$$2CHOO^{\bullet} \begin{cases} \rightarrow C=O^{*} + COH + O_2\,{}^3S_g^{-} \\ \rightarrow C=O + COH + O_2\,{}^1D_g \text{ or dimols } (O_2)_2^{*} \end{cases}$$

The radiative deactivation of the excited molecules leads to UL:

$$C=O^{*} \rightarrow C=O + h\nu, \lambda = 420-560\,nm$$

$$(O_2)_2^{*}({}^1\Delta_g\,{}^1\Delta_g)_{v=0} \begin{cases} \rightarrow 2O_2({}^3S_g^{-})_{n=0} + h\nu, & \lambda = 634\,nm \\ \rightarrow 2O_2({}^3\Sigma_g^{-})_{v=1} + h\nu, & \lambda = 703\,nm \end{cases}$$

The red emission predominant over the blue-green one (420-560 nm) observed from the aerobically incubated bull spermatozoa and liposomes of egg phospholipids (Fig. 8A and B) is in agreement with the above-proposed mechanism. The enhancement of spontaneous and Fe^{2+}-induced UL of spermatozoa from different species by Fe^{2+} ions, pure molecular oxygen and the Fe^{2+}-ascorbate prooxidative system observed in the experiment on oxidative (chemical) stress (Fig. 6 and 7) also support this mechanism. The redox-cycling Fe^{2+}-ascorbate system has been commonly used as a prooxidant. When suspension of washed, motile, domestic-animal spermatozoa was incubated aerobically in the presence of Fe^{2+}-ascorbate, nearly all spermatozoa lost their motility after a short incubation time (1-2 h) (Mann, 1982). It has been established that lipid peroxides and products of their decomposition such as, e.g. malonaldehyde are highly spermicidal: they damage not only the sperm plasmolemma and outer acrosomal membrane, but in addition, mitochondrial sheath and axial filament. This damage results in a fast and irreversible immobilization of sperm cells. Our results on the correlation between I_{max}, dI/dt, τ_{max} and motility and vitality of spermatozoa presented in Fig. 3, 6 and 7 are in agreement with the above findings. The effect of temperature and prolonged incubation time on UL and motility and vitality described in the last chapter, is also consistent with the interpretation offered.

It is important to realize that spermatozoa were separated from the seminal fluid (plasma) in our experiments. This fluid contains a variety of antioxidants, e.g. ascorbic acid (0.14 mg/ml), chelating and reducing agents, amino-acids and proteases (Mann, 1982; Vishwanath, Munday, Curson & Shannon, 1992). These substances play a beneficial role in sperm survival; their action is believed to depend primarily on free radical scavenging and metal binding capacity. It has been well established that an excessive dilution of semen exerts a deleterious effect on spermatozoa cells. Washed spermatozoa are much more sensitive to endogenous and exogenous lipid peroxidation than those in unwashed sperm (Mann, 1982; Strzęzek, Luberda & Demianowicz, 1992, Viswanath *et al.*, 1992). The effect of formaldehyde on spermatozoa is quite different from the behaviour of e.g. yeast cells (Rajfur, 1994) or higher plant cells (Slawinska & Polewski, 1987) which show a high sensitivity towards this compound. A high value of X for motility is probably associated with the denaturation of contractible proteins by formaldehyde. UL response is related to control mechanisms of homeostasis (feedback) and, like vitality, is less sensitive to formaldehyde than motility. Therefore, the weak effect of formaldehyde on UL of bull spermatozoa (see Table 2) can be explained by the protective action of the medium containing an egg yolk. For this reason, under our experimental conditions, the use of washed cells may facilitate the peroxidation of lipids in membranes and accumulation of hydroperoxides. Our research suggests that UL of spermatozoa results from radical reactions localized in membranes, such as, lipid peroxidation. This interpretation adheres to a commonly-accepted hypothesis of "imperfections", according to which UL results from rare and random aberrations in the energetic and metabolism of a living system,

e.g. leakage of electrons from the respiratory chain.

An important question arises: what is the absolute intensity (I_a) of UL and the cell specific intensity (I_a/cell) emitted from intact, unperturbed spermatozoa? Calculation of the correct value requires complex corrections according to the formula:

$$I_a^{'} = n \cdot 10^{Ad} \cdot \Phi \cdot \eta / t \cdot n_s ,$$

where n is the number of photo-counts per time interval t, A is absorption (optical density) of the cell suspension, d is mean path length of UL (half the diameter of the height of suspension), $\hat{O}$ is the mean quantum efficiency of the photomultiplier photo-cathode in the spectral range of UL (300-825 nm), η is the photon collection coefficient dependent on the geometry and refractive index of the detector-cuvette system, and n_s is the number of the cells. Based on these corrections, we estimated the absolute value of cell-specific UL at about 0.0007 photons/s per spermatozoon. Thus, the probability of spontaneous emission from unperturbed cells is very low: more than a thousand cells are needed to generate one photon during 1 s. It seems interesting to compare this value with cell-specific spontaneous UL ($I_a^{'}$) from other cells. Van Vijk and Van Aken (1992) reported that rat hepatocytes emit 0.02 photons/s per cell, while Cadenas, Wefers and Sies (1981) found this number to be only 30 photons/s per 10^6 cells. Tumor samples emit 300±90 photons/min per cm^2 and the normal ones 20±6 photons/s per cm^2 (Grasso, Grillo, Musumeci, Triglia, Rodolica, Cammisuli, Rinzivillo, Fragati, Santuccio & Rodolico, 1992). The absolute value of cell-specific UL ($I_a^{'}$) increases 30-50 times under stress conditions. For example, the UL emission per bull spermatozoon incubated in oxygenated solutions containing Fe^{2+} or Fe^{2+}-ascorbate is equal $I_a^{'}=(2.8\pm2)\cdot10^{-2}$ photons/s. Within the same order of magnitude are $I_a^{'}$ values for ram and bull spermatozoa subjected to an oxidative or osmotic stress.

The irradiation of bull spermatozoa with white light results in a delayed UL that has the cell-specific intensity (I_a/cell) about $(3.0\pm1.7)\cdot10^{-2}$ photons/s per cell. It is worth mentioning that this phenomenon is characteristic, not only of living intact spermatozoa, but also of dead cells. In the letter case, the I_a/cell value is 11 times lower in comparison with surviving cells. Van Wijk and Van Aken (1991) also found that delayed UL does not require intanct cells. In our experiments, however, the phenomenological relationship between the intensity (I) of the late stationary phase of delayed UL and successive irradiation cycle exists only for living cells. A similar result was observed by Musumeci, Triglia, Grasso, Scordino and Sitko (1994) for germinating soya seeds. A consistent explanation for the delayed UL of spermatozoa is currently lacking. It was reported that the photo-induced UL was not retained in the cytoplasmatic fraction after fractionation of mammalian cells (hepatocytes, fibroplasts). Instead, the delayed UL was found in the nuclei fraction (Van Wijk & Van Aken, 1991; Van Wijk, Van Aken, Mei & Popp, 1993). Therefore, these authors suggest that differences in the chromatin structure may explain the cell-specific UL. In this model, called "coherence hypothesis", it is assumed that electronically-excited states with long lifetime are generated within the DNA of nuclear chromatin, and that there is a negative feedback loop in living cells, which couples together states of the coherent "biophoton" field and the conformational states of the DNA, and thus also metabolic processes in the cell (Nagl & Popp, 1983; Popp, Nagl, Li, Scholtz, Weingaertner & Wolf, 1984; Popp *et al.*, 1992). Spermatozoa cells containing a large amount of chromatin might be a suitable object for testing the "coherence hypothesis". Any environmental chemical of physical factor, however, affecting the nuclear chromatin and DNA at first interacts with the membrane of cells and/or organelles. This interaction can perturb the homeostasis and metabolism of a cell, thus affecting the emitted UL. In order to discriminate between UL originating from DNA and that from membranes, it seems necessary to learn more about primary interactions occurring in the membranes. Alternative explanations suggest endogenous porphyrins and/or cytochroms (Lubart *et al.*, 1992) or flavins (Ezzahir, Godlewski, Kwiecinska, Rajfur, Slawinski & Scieszka, 1990) as primary targets for the light-spermatozoa cell interaction. Thus, spectral analysis of the excitation of spermatozoa cells and model systems, e.g. DNA, DNA-histones or chromatin is necessary in order to clearly understood the phenomenon.

CONCLUSIONS

The results of our research provide unequivocal evidence for the existence of spontaneous and

stress-induced UL of spermatozoa from bull, ram and boar.

Roughly-estimated cell-specific intensity (I_s/cell) is very low, $7 \cdot 10^{-4}$ for intact cells in oxic conditions and $2 \cdot 10^{-2}$ for perturbed cells in a hyperoxic environment. The spectral region of the Fe^{2+}-induced UL covers wavelengths 300-850 nm with about 70% of emission in the red (λ>600 nm).

Environmental detrimental stress factors such as osmotic pressure, hypotonic shock, strong light, physiologically high temperature, hyperoxic conditions and pro-oxidant substances enhance the intensity and light sum with concomitant decrease of motility and vitality of the cells tested. In the case of Fe^{2+}-induced UL from bull spermatozoa subjected to osmotic stress, such parameters as I_{max}, τ_{max} and incubation time correlate with physiological indices of motility and vitality. Thus, the light emission is functionally linked to the metabolism and regularoty processes taking place in this important class of generative cells. Moreover, the correlation between parameters of UL and physiological indices can be used as a new diagnostic tool for the evaluation of sperm viability.

The data are in favor of the mechanisms of membrane lipid peroxidation as a source of chemi-excitation. The cell membrane is a primary target for stress factors which accelerate exergonic radical reactions of lipids peroxidation that produce excited molecules and photons. However, other sources of UL of spermatozoa cells are possible, especially in the case of light-induced delayed UL. One cannot exclude the possibility that the energy state and structure of chromatin in sperm cells may be changed by membrane perturbations and products of lipid peroxidation. This possibility deserves closer examination in further research.

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