

FERROUS ION INDUCED PHOTON EMISSION OF RABBIT SPERMATOZOA*

Piotr Gogol

Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland

Abstract

Ultraweak photon emission measurements were found to be a useful tool for investigating the influence of stress factors on living cells. In the present study, induced photon emission of rabbit spermatozoa was investigated using a luminometer. The relationship of emission intensity and kinetics to sperm concentration in the sample, to Fe(2+) ions concentration in the photon emission inducing solution and to pH of medium were analysed. Sperm concentration in the analysed sample was shown to affect the intensity and kinetics of emission. As sperm concentration in the sample increased, an increase was observed in intensity of emission. It was found that the relationship for Fe(2+) ions concentration and emission intensity exhibits a bell-shaped curve and achieves a maximum at 0.025 mM. Photon emission intensity increases with increasing pH of the medium. The results prove the possibility of measuring induced photon emission of rabbit spermatozoa with the use of a luminometer and determine the optimal conditions of its measurement.

Key words: rabbit, semen quality, photon emission, lipid peroxidation

The possibility of determining both fresh and conserved semen quality *in vitro* is of major importance to artificial insemination practice. The classical methods used to evaluate semen quality allow only one characteristic, structural property or element of sperm metabolism to be investigated. In general, they measure the sperm cell concentration, the progressive motility, the percentage of viable cells and the morphology. Moreover, the results obtained using these methods correlate only to a certain degree with the fertilizing capacity of spermatozoa (Xu et al., 1998; Rodriguez-Martinez, 2003; Gadea, 2005). This calls for new research methods that can be used to determine semen quality.

A promising methodological solution in this area is the use of photon emission (luminescence) measurements. Measurements of the spectral distribution of sperm emission and analysis of the relationships between the concentration of Fe ions and intensity of induced luminescence show that it is strictly related to lipid peroxidation

*This work was conducted as part of NRIAP statutory activity, project no. 3423.1.

(Sławiński et al., 1998; Gogol, 2005), which is one of the basic biochemical processes accelerating the ageing of spermatozoa, especially during semen conservation (Cecil and Bakst, 1993; Cerolini et al., 2000; Castellini et al., 2000). In lipid peroxidation, which is a chain free radical process, the generated reactive oxygen species, by initiating further peroxidation reactions, can cause serious damage to the cells and significantly change their structure and metabolism. Reactive oxygen species has been shown to cause membrane deterioration, resulting in ATP depletion (de Lamirande and Gagnon, 1992; Armstrong et al., 1999), decreased sperm movement (Aitken et al., 1993 a; Armstrong et al., 1999), DNA damage (Donnelly et al., 1999) and blocked sperm-egg fusion (Mammoto et al., 1996).

At present, the most widely used assay for lipid peroxidation involves the measurement of malondialdehyde (MA), a small molecular mass degradation product of peroxidative process that can be measured by virtue of its capacity to form adducts with thiobarbituric acid (Aitken et al., 1993 b). Although the method is sensitive and can detect the end-point reaction product of lipid peroxidation, it is relatively elaborate and provides only an indirect measure of lipid peroxidation.

Chemiluminescence is considered to be an alternative and sensitive method to assess the oxidation or autooxidation of lipids (Miyazawa et al., 1994; Albertini and Abuja, 1998). In several studies, the luminescence signal has been correlated with other indicators of lipid peroxidation, such as the MDA concentration (Doi et al., 2002), the concentration of exogenously added lipid hydroperoxides (Guajardo et al., 2002), and the content of conjugated dienes (Albertini and Abuja, 1998). In the case of spermatozoa, however, the intensity of spontaneous luminescence is extremely weak and thus difficult to measure. Earlier studies (Laszczka et al., 1995; Sławiński et al., 1998; Gogol, 2005; Gogol and Szczęśniak-Fabiańczyk, 2006) show that recording the ferrous ion induced luminescence can be an alternative and relatively simple method of detecting and quantifying lipid peroxidation damage and oxidative stress in boar and ram spermatozoa cells. Moreover, the relationship between induced photon emission of spermatozoa and their physiological state (ATP level, motility) and sperm MDA production indicate that luminescence assessment may be useful as an objective laboratory test of semen quality (Gogol et al., 2007; Gogol and Pieszka, 2008; Gogol et al., 2009). However, ultraweak photon emission of rabbit spermatozoa has not been investigated.

This study was designed to determine the possibility of using a luminometer for analysing ultraweak photon emission of rabbit spermatozoa and to determine the optimal conditions of its measurement, allowing for emission with the maximum ratio of the registered signal to the background (equipment noise).

Material and methods

Semen collection and dilutions

Ten adult, healthy New Zealand White rabbits were used in this study. Semen was collected in May and June using an artificial vagina. Directly after collection

sperm motility and concentration was determined. Ejaculates used in the experiment had $260\text{--}720 \times 10^6$ sperm/ml of concentration and 60–80% of motility. Because of the relatively large number of spermatozoa required for luminescence measurement, ejaculates from several males were mixed together. Semen samples were then diluted with 0.9% NaCl solution and centrifuged twice (15 min/600 g) to separate sperm from plasma. Separated spermatozoa were diluted with 0.9% NaCl solution to the required concentration. Directly after spermatozoa dilution luminescence measurements were performed.

Luminescence measurements

Luminescence was measured at 20°C using an AutoLumat LB953 (Berthold, Bad Wildbad, Germany) luminometer equipped with a cooled photomultiplier with a spectral response range from 370 to 620 nm. To 700 μl of the washed sperm suspension 10 μl of 5 mM luminol (as luminescence enhancer) solution was added. Emission was induced by adding (using automated injector system) 100 μl FeSO_4 solution to the sample. Immediately after injection light emission kinetics was measured during 500 seconds.

Effect of sperm concentration in the analysed sample on luminescence intensity and kinetics

Spermatozoa separated from the plasma were diluted with 0.9% NaCl to concentrations of 50, 100, 200 or 300×10^6 sperm/ml. A sample of 700 μl sperm suspension containing 10 μl luminol solution (5 mM) was placed in the luminometer. Then 100 μl 0.2 mM FeSO_4 solution (final concentration 0.025 mM) was added and luminescence kinetics was measured. This experiment was conducted using spermatozoa derived from 10 bucks.

Effect of ferrous sulfate on luminescence intensity

To a 700 μl plasma-free sample with sperm concentration of 200×10^6 sperm/ml containing 10 μl luminol solution (5 mM) was added 100 μl FeSO_4 solution with a concentration of 0.05, 0.1, 0.2, 0.4 or 0.8 mM and luminescence was measured. Ferrous ion stock solution (4 mM) in distilled water was freshly prepared before each experiment. This experiment was repeated in triplicate using semen from 9 bucks and results were expressed as means.

Effect of pH on luminescence intensity and kinetics

Spermatozoa separated from the plasma were diluted to a concentration of 200×10^6 sperm/ml with 0.9% NaCl with pH of 6.0, 7.0, 8.0 or 8.5. The pH of a medium was adjusted with 1 M NaOH. To a 700 μl spermatozoa suspension containing 10 μl luminol solution (5 mM) was added 100 μl FeSO_4 solution with a concentration of 0.2 mM and luminescence was measured. Spermatozoa from 6 bucks were evaluated in this experiment.

Results

Sperm concentration in the analysed sample was shown to influence luminescence intensity and kinetics. As sperm concentration in the sample increased, an increase was observed in intensity of luminescence (total pulses measured). With a higher concentration of sperm in the sample, luminescence intensity increased more rapidly and after reaching the maximum (peak max), underwent a decline (Figure 1). Maximum luminescence intensity was higher and more pronounced in samples with higher sperm concentration.

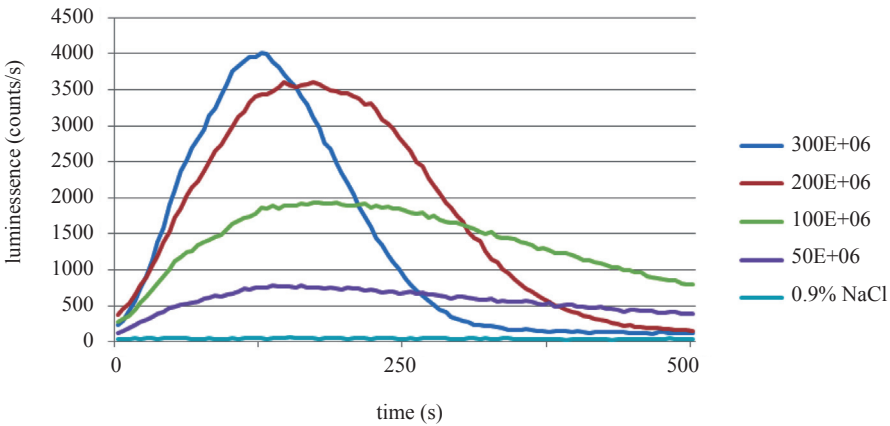


Figure 1. Effect of sperm concentration in a sample on kinetics of induced luminescence

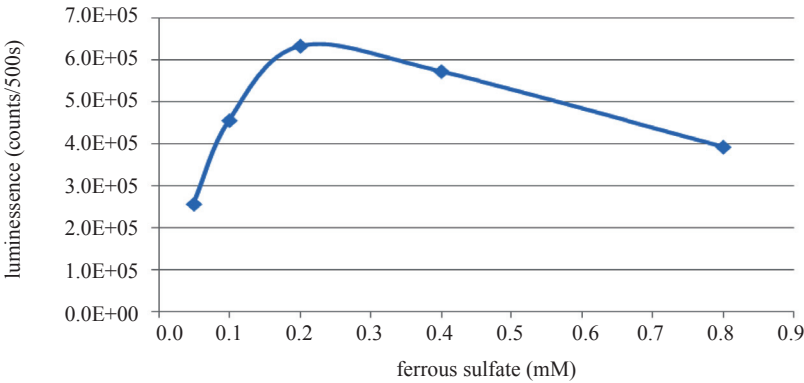


Figure 2. Effect of added ferrous sulfate concentration on intensity of luminescence of rabbit spermatozoa

It was found that the curve depicting the relationship between $\text{Fe}(2+)$ ions concentration and induced luminescence intensity exhibits a bell shape and achieves a maximum at a final concentration of 0.025 mM FeSO_4 (Figure 2).

The intensity and kinetics of the luminescence showed a strong pH dependence. The increase in pH (within the analysed range of 6.0–8.5) was paralleled by an increase in peak height and reduced emission time (Figure 3). Photon emission intensity increases with increasing pH of the medium. Luminescence intensity reached the maximum value when the pH was 8.5. Because pH 8.0 and 8.5 is above a physiological level, pH 7.0 of 0.9% NaCl solution was selected for subsequent research.

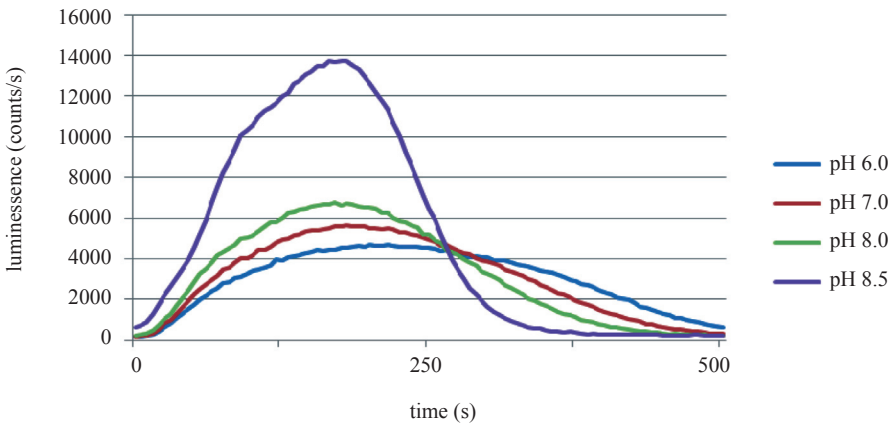


Figure 3. Effect of pH on induced luminescence of spermatozoa

Discussion

These results support previous studies which demonstrated that spontaneous photon emission intensity from spermatozoa is very low, but could be significantly enhanced by the presence of ferrous ions. The minimal spontaneous photon emission observed in sperm populations incubated in the absence of ferrous ions suggests that if transition metals, mainly $\text{Fe}(2+)$, are not available, lipid radical formation is maintained at a very low level in these cells. This reflects the fact that mammalian spermatozoa possess an active defence against lipid peroxidation in the form of the glutathione peroxidase (GPx)/glutathione reductase system (Alvarez and Storey, 1989; Williams and Ford, 2005). The classical GPx1 works in tandem with phospholipase A_2 to detoxify free lipid peroxides liberated from phospholipids. In addition, spermatozoa possess a form of phospholipid hydroperoxide, GPx4, that can detoxify membrane lipid peroxides *in situ*, without the need for prior phospholipase A_2 (PLA $_2$) action (Tramer et al., 2004). In addition to the GPx system, lipid peroxides

may be stabilized in the sperm plasma membrane by chain-breaking antioxidants such as vitamin E (Sheweita et al., 2005; Drevet, 2006). However, in the presence of ferrous ions, these peroxides are induced to break down with the generation of alkoxy radicals ($RO\cdot$). These radicals may then attack existing lipids or lipid peroxides in the membrane to generate a mixture of alkoxy and peroxy radicals which appear to be important in the propagation of the chain reaction of lipid peroxidation in the sperm membrane (Aitken et al., 1993 b; Alvarez and Storey, 1995). In these radical chain reactions, electron-excited molecules are generated and then radiatively deactivated, which manifests itself as an emission of light (chemiluminescence, ultraweak photon emission).

In previous studies, ferrous ion promotion was found to be the most effective means of triggering a peroxidative response in spermatozoa cells (Jones et al., 1979; Aitken et al., 1993 a, b). Ferrous ions are clearly much more effective than ferric ions in the catalysis of lipid peroxidation in human spermatozoa. Since there is no evidence for hydroxyl radical ($OH\cdot$) formation when spermatozoa are exposed to $Fe(2+)$ (Aitken et al., 1993 b), the dependence of photon emission intensity on the presence of ferrous ions would not appear to be associated with the first chain initiation of the lipid peroxidation cascade. Rather, as indicated earlier, $Fe(2+)$ induces the breakdown of pre-existing lipid peroxides to create the alkoxy and peroxy radicals responsible for photon emission. The fact that $Fe(3+)$ is not effective in this regard suggests that there is insufficient superoxide anion ($O_2^{\cdot-}$) being generated by normal spermatozoa to bring about the first step in the Haber–Weiss reaction, which involves the $O_2^{\cdot-}$ –mediated reduction of $Fe(3+)$ to $Fe(2+)$. The lack of activity on behalf of $Fe(3+)$ may also reflect the fact that reactions of ferric ions with lipid peroxides are slower than those recorded with $Fe(2+)$. $Fe(3+)$ may also be unable to form the appropriate complexes within spermatozoa, since the specific nature of $Fe(3+)$ chelates makes a significant difference to the reaction rates observed in the promotion of lipid peroxidation (Halliwell and Gutteridge, 1990). In this context, it may be particularly important that spermatozoa are coated with lactoferrin (Goodman and Young, 1981; Jin et al., 1997), which binds two atoms of $Fe(3+)$ per molecule in a manner that does not support the catalysis of peroxy radical formation.

The results of this study point to the importance of a rigorous control of pH if luminol-dependent luminescence measurements are to be made. The strong dependence of the luminescence intensity on pH can be attributed to the fact that luminol, one of the most often used classical chemiluminescence agents exhibits the emission of maximum intensity in alkaline medium. The luminol-dependent chemiluminescence intensity is increased by the increasing pH of medium and is dependent on the used catalyst being either transition metal or biocatalyst containing hemo-group.

Considering the association between induced luminescence and lipid peroxidation, the results of this study confirm the possibility of using the measurements of this biophysical phenomenon to assess the intensity of pathological oxidative processes in rabbit spermatozoa. It can be conjectured that similar to ferrous ion-promoted malondialdehyde assay (as an indicator of lipid peroxidation), the Fe ion-induced photon emission provides information on two aspects of sperm biochemistry: the extent to which oxidative stress has led to the accumulation of lipid peroxides in

the membrane which will respond to the presence of ferrous ions by stimulating the propagation of a peroxidative chain reaction, and/or the ability of the spermatozoon to withstand the propagation of lipid peroxidation through the presence of chain-breaking antioxidants, defensive enzymes and transition metal chelators. Regardless of which mechanism is involved, luminol-dependent chemiluminescence allows not only for determining the level of reactive oxygen species (generated during the lipid peroxidation process), it also permits the observation of the reaction kinetics. This makes it possible to obtain more detailed information about the processes that take place in the sperm cells and how to use it for semen quality evaluation.

In conclusion, the present findings prove the possibility of analysing induced luminol-dependent luminescence of rabbit spermatozoa with the use of a luminometer. The spontaneous rates of photon emission in the spermatozoa specimens were low, but could be significantly enhanced by the presence of a ferrous ion as a promoter. In this promoted form, the analysis of photon emission can be useful for monitoring oxidative damage to rabbit spermatozoa.

References

- Aitken R.J., Harkiss D., Buckingham D.W. (1993 a). Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J. Reprod. Fertil.*, 98: 257–265.
- Aitken R.J., Harkiss D., Buckingham D.W. (1993 b). Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol. Reprod. Dev.*, 35: 302–315.
- Albertini R., Abuja P.M. (1998). Monitoring of low-density lipoprotein oxidation by low-level chemiluminescence. *Free Radic. Res.*, 29: 75–83.
- Alvarez J.G., Storey B.T. (1989). Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.*, 23: 77–90.
- Alvarez J.G., Storey B.T. (1995). Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol. Reprod. Dev.*, 42: 334–336.
- Armstrong J.S., Rajasekaran M., Chamulitrat W., Gatti P., Hellstrom W.J., Sika S.C. (1999). Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic. Biol. Med.*, 26: 869–880.
- Castellini C., Lattaioli P., Moroni M., Minelli A. (2000). Effect of seminal plasma on the characteristics and fertility of rabbit spermatozoa. *Anim. Reprod. Sci.*, 63: 275–282.
- Cecil H.C., Bakst M.R. (1993). *In vitro* lipid peroxidation of turkey spermatozoa. *Poult. Sci.*, 72: 1370–1378.
- Cerolini S., Maldjian A., Surai P., Noble R. (2000). Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim. Reprod. Sci.*, 58: 99–111.
- Doi H., Iwasaki H., Masubuchi Y., Nishigaki R., Horie T. (2002). Chemiluminescence associated with the oxidative metabolism of salicylic acid in rat liver microsomes. *Chem. Biol. Interact.*, 140: 109–119.
- Donnelly E.T., McClure N., Lewis S.E. (1999). The effect of ascorbate and alpha-tocopherol supplementation *in vitro* on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis*, 14: 505–512.
- Drevet J.R. (2006). The antioxidant glutathione peroxidase family and spermatozoa: a complex story. *Mol. Cell. Endocrinol.*, 16: 70–79.
- Gadea J. (2005). Sperm factors related to *in vitro* and *in vivo* porcine fertility. *Theriogenology*, 63: 431–444.
- Gogol P. (2005). Iron-induced luminescence of boar spermatozoa cells. *Ann. Anim. Sci.*, 5: 12–17.
- Gogol P., Pieszka M. (2008). Ferrous ion induced photon emission as a method to quantify oxidative stress in stored boar spermatozoa. *Folia Biol. (Krakow)*, 56: 173–177.

- Gogol P., Szczęśniak-Fabiańczyk B. (2006). Effect of long-term storage on induced photon emission of boar spermatozoa. *Czech J. Anim. Sci.*, 51: 61–65.
- Gogol P., Szczęśniak-Fabiańczyk B., Wierzchoś-Hilczer A. (2009). The photon emission, ATP level and motility of boar spermatozoa during liquid storage. *Reprod. Biol.*, 9: 39–49.
- Gogol P., Wierzchoś-Hilczer A., Cegła M. (2007). Iron-induced luminescence as a method for assessing lipid peroxidation of frozen-thawed goat spermatozoa. *Animal*, 1: 844–848.
- Goodman S.A., Young L.G. (1981). Immunological identification of lactoferrin as a shared antigen on radioiodinated human seminal plasma. *J. Reprod. Immunol.*, 3: 99–108.
- Guajardo M.H., Terrasa A.M., Catala A. (2002). Retinal fatty acid binding protein reduce lipid peroxidation stimulated by long-chain fatty acid hydroperoxides on rod outer segments. *Biochim. Biophys. Acta*, 1581: 65–74.
- Halliwell B., Gutteridge J.M. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods. Enzymol.*, 186: 1–85.
- Jin Y.Z., Bannai S., Dacheux F., Dacheux J.L., Okamura N. (1997). Direct evidence for the secretion of lactoferrin and its binding to sperm in the porcine epididymis. *Mol. Reprod. Dev.*, 47: 490–496.
- Jones R., Mann T., Sherins R.J. (1979). Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil. Steril.*, 31: 531–537.
- Lamirande E. de, Gagnon C. (1992). Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J. Androl.*, 13: 379–386.
- Laszcza A., Godlewski M., Kwiecińska T., Rajfur Z., Sitko D., Szczęśniak-Fabiańczyk B., Sławiński J. (1995). Ultraweak luminescence of spermatozoa. *Curr. Top. Biophys.*, 19: 20–31.
- Mammoto A., Masumoto N., Tahara M., Ikebuchi Y., Ohmichi M., Tasaka K., Miyake A. (1996). Reactive oxygen species block sperm-egg fusion via oxidation of sperm sulfhydryl proteins in mice. *Biol. Reprod.*, 55: 1063–1068.
- Miyazawa T., Fujimoto K., Usuki R. (1994). Rapid estimation of peroxide content of soybean oil by measuring thermoluminescence. *J. Am. Oil Chem. Soc.*, 71: 343–345.
- Rodriguez-Martinez H. (2003). Laboratory semen assessment and prediction of fertility: still utopia? *Reprod. Domest. Anim.*, 38: 312–318.
- Sheweita S.A., Tilmisany A.M., Al-Sawah H. (2005). Mechanisms of male infertility: role of antioxidants. *Curr. Drug Metab.*, 6: 495–501.
- Sławiński J., Godlewski M., Gumińska M., Kędryna T., Kwiecińska T., Laszcza A., Szczęśniak-Fabiańczyk B., Wierzuchowska D. (1998). Stress-induced peroxidation and ultraweak photon emission of spermatozoa cells. *Curr. Top. Biophys.*, 22: 195–203.
- Tramer F., Caponecchia L., Sgrò P., Martinelli M., Sandri G., Panfili E., Lenzi A., Gandini L. (2004). Native specific activity of glutathione peroxidase (GPx-1), phospholipid hydroperoxide glutathione peroxidase (PHGPx) and glutathione reductase (GR) does not differ between normo- and hypomotile human sperm samples. *Int. J. Androl.*, 27: 88–93.
- Williams A.C., Ford W.C. (2005). Relationship between reactive oxygen species production and lipid peroxidation in human sperm suspensions and their association with sperm function. *Fertil. Steril.*, 83: 929–936.
- Xu X., Pommier S., Arbov T., Hutchings B., Sotto W., Foxcroft G.R. (1998). *In vitro* maturation and fertilization techniques for assessment of semen quality and boar fertility. *J. Anim. Sci.*, 76: 3079–3089.

PIOTR GOGOL

Indukowana jonami żelaza emisja fotonowa plemników królika

STRESZCZENIE

Przy użyciu luminometru badano indukowaną emisję fotonową (luminescencję) plemników królika. Oceniano zależność natężenia i kinetyki emisji od koncentracji plemników w próbce, stężenia jonów żelaza w roztworze indukującym oraz pH medium. Wykazano wpływ koncentracji plemników w badanej próbce na natężenie i kinetykę emisji fotonowej. Wraz ze wzrostem koncentracji plemników następował wzrost wysokości pików i skracanie czasu emisji. Stwierdzono, że w przypadku emisji indukowanej jonami żelaza, krzywa zależności pomiędzy stężeniem jonów żelaza a natężeniem luminescencji przyjmuje kształt dzwonu, z maksimum przy stężeniu 0,025 mM FeSO_4 . Natężenie emisji fotonowej wzrastało wraz ze wzrostem pH medium. Uzyskane wyniki dowodzą możliwości badania indukowanej emisji fotonowej plemników królika przy użyciu luminometru i określają optymalne warunki jej pomiaru.