Autoradiographically Detected Impairment of RNA Synthesis Pattern in 8–16 Cell Bovine Embryos After Irradiation

J. Pivko[1], E. Kubovičová[1], P. Grafenau[1], M. Zibrín[2] and O. Chlebovský[3]

1 *Research Institute of Animal Production, Institute of Genetics and Reproduction of Animals, Nitra, Slovakia*
2 *University of Veterinary Medicine, Department of Anatomy and Histology, Košice, Slovakia*
3 *Faculty of Science, P. J. Šafárik University, Department Zoology and Ecology, Košice, Slovakia*

Abstract. Early preimplantation bovine embryos at 8- or 16-cell stage were analysed by [5-^{3}H]uridine autoradiography for distribution of newly synthesized RNA after ^{60}Co irradiation with a single dose of 1 Gy, 2 Gy or 4 Gy gamma rays, respectively. Embryos irradiated with a single dose of 1 Gy showed equally decreased synthesis of RNA in nucleoplasma as well as in nucleolus. In embryos irradiated with a single dose of 2 Gy or 4 Gy, RNA synthesis was decreased and localized mostly on the periphery of the nucleus; in both cases of irradiation, the nucleus center being without labelling. In most of embryos irradiated with a dose of 4 Gy, the nucleoli were not labelled, and an increasing occurrence appeared of various nucleus chromatin segregation forms, mainly as its marginalization.

Key words: Bovine — Early embryo — Irradiation — RNA — Synthesis

Introduction

Critical point in embryonic development is known as the maternal-zygotic transition when major transcriptions from the embryonic genome are initiated (Telford et al. 1990). This stage varies among mammalian species and it is situated at the 8- to 16-cell stage in bovines. This is also the stage of development when embryos often cease to develop under suboptimal *in vitro* culture conditions (De Sousa et al. 1998). In early embryos produced by different biotechnologies, deviations from the normal pattern of differentiation in the nuclear and nucleolar microarchitecture have been detected. In bovine embryos produced *in vitro* from oocytes isolated from

Correspondence to: Prof. MVDr. Juraj Pivko, DrSc., Research Institute of Animal Production, Institute of Genetics and Reproduction of Animals, Hlohovská 2, 949 92 Nitra, Slovakia. E-mail: pivko@vuzv.sk

different size categories of antral follicles, the degree of extranucleolar chromatin condensation and the association of the nucleolus-precursor-body (NPB) with perinucleolar chromatin was altered as well as the autoradiographically established sterical pattern of RNA synthesis (Pavlok et al. 1993). In embryos produced by nuclear transfer, the development of the NPB as well as RNA synthesis was arrested for three consecutive cleavages (Kaňka et al. 1991). A similar test for autoradiographically detectable intranuclear localization of RNA synthesis was performed on bovine embryos chronically irradiated in the Chernobyl area (Pivko et al. 1997). These observations show that otherwise the expected radiation injury of the genome does not probably influence markedly the early events of the developing embryo in the same way as in Pavlok's study and that the aberrant cytoplasmic command of the nuclear events known in other types of early bovine embryo impairment (Pavlok et al. 1993) is not seen in early embryos collected from the chronically irradiated animals. We suppose, that the pattern of embryonic RNA synthesis onset within embryo nucleus may represent a generally applicable methodology for testing the embryonic genome integrity as suggested by Kopečný and Niemann (1993).

Materials and Methods

Early 8- or 16-cell preimplantation embryos were collected from superovulated Holstein-Friesian (HF) cows after slaughter. Superovulation was induced with FSH (Folicotropin, Léčiva, Prague, Czech Republic) over 4 days by a total dose of 24 mg, i.m. (4.4; 4.4; 2.2; 2.2 mg) and estrus was synchronized by PGF_2 alpha (Remophan, Léčiva, Prague, Czech Republic) in a dose of 3 ml i.m. (Grafenau et al. 1988). Isolated tubae and uteri were flushed with a culture medium Dulbecco PBS (Bioveta, Ivanovice na Hané, Czech Republic) supplemented with 1 % bovine serum. The embryos were then evaluated under a dissecting microscope and categorized according to general criteria of development competence and quality evaluation (Stringfellow and Seidel 1990) and morphologically normal embryos were further treated as follows: the embryos (8- and 16-cell) were cultured *in vitro* for 5 h in a flushing medium (Medi Cult a/s, Copenhagen, Denmark) supplemented with 8 mg/ml BSA at 38.5 ℃ (5 % CO_2 in the air). The embryos were then irradiated with a single dose of 1 Gy, 2 Gy or 4 Gy, respectively, with a dose rate of 0.19 Gy/min at 38 ℃ using a therapeutical irradiating apparatus CHISOSTAT with a source of gamma ^{60}Co radiation. After irradiation the embryos were briefly washed and cultured *in vitro* for 16 h in the culture medium M-199 (Pavlok et al. 1993) supplemented with 20 % heat-inactivated estrus cow serum (ECS) at 38.5 ℃ in an atmosphere composed of 5 % CO_2, 10 % O_2, 85 % N_2 and subsequently processed for autoradiography.

After 16 h of *in vitro* culture the irradiated embryos were transferred into M-199 medium (Pavlok et al. 1993) supplemented with [5-^{3}H] uridine (ÚVVVR, Prague, Czech Republic; specific activity 740 GBq/mmol) in a dose of 100 μCi/ml and cultured for 20 min. After culture with [5-^{3}H]uridine the embryos were briefly washed by passage through the culture medium, and then immediately fixed with

4% paraformaldehyde (Merck, Darmstadt, Germany) in Sörensen buffer at pH 7.4, dehydrated by ethanol and embedded in LR White resin. The blocks with embryos were cut on a LKB-Nova ultramicrotome. Serial semi-thin sections (1 μm) were used for light microscopy autoradiography. Slides with embryo sections were coated with liquid nuclear emulsion Ilford K.5, exposed for 38 days and stained with Methylene Blue (Gurr) after development with D.19. The pattern of labelling was evaluated by light microscopy (magnification: 500 and 1000), included arbitrary criteria allowing evaluation of both level and labelling localization in nucleoplasma and nucleus of experimentally irradiated 8- and 16-cell bovine embryos.

The labelling pattern of individual nuclei within embryos as well as the labelling intensity were classified using a score ranging from 0.0 to 1.0 according to the amount and localisation of silver grains in nuclei and nucleoli (Table 1):

Category 0: No labelling in nuclei and nucleoli; arbitrary activity; score 0.0.

Category 1: Low labelling intensity of the periphery of nucleus; score 0.1.

Category 2: Weak labelling mostly localized at the periphery of the nucleus; score 0.3.

Category 3: Intensive homogenous labelling in nuclei and nucleoli; score 0.7.

Category 4: Very high labelling at the periphery and throughout the nucleus and nucleolus; score 1.0.

The category score was calculated by multiplying the number of nuclei classified in each class by the corresponding scoring factor (Hrudka 1979).

The results of nuclear labelling are expressed as the index of activity (I) according to the formula:

Activity index (I) = total score/number of nuclei counted.

Table 1. Nuclear incorporation of [5-^{3}H]uridine in 8- to 16-cell bovine embryos 16 h after irradiation with a ^{60}Co source

Dose of irradiation	No. of embryos	No. of nucleus sections with a diameter > 5	Category of distribution of nuclear labelling[b]					No. of blastomeres		Activity index[c]
			0	1	2	3	4	normal	degen. and fragm.	
0	5	195	–	6	36	99	54	195	–	0.690
1 Gy	5	223	–	16	79	128	–	223	–	0.515
2 Gy	5	214	5	112	97	–	–	146	68	0.188
4 Gy	5	221	9	141	72	–	–	169	152	0.161

[a] Evaluated by light microscopy autoradiography of semithin sections of 8- to 16-cell bovine embryo nuclei of a diameter > 5 μm. [b] An arbitrary scale combining both localization and labelling intensity according to Pavlok et al. (1993). [c] Activity index (I) = total score/number of nuclei counted.

Results

Early bovine embryos selected for irradiation were prevailingly at the end of 8-, and 16-blastomere stage, corresponding to the beginning of the compaction period. *Zona pellucida* of embryos was intact, blastomeres were regularly developed and periviteline space was transparent without fragments of cells.

The morphology of blastomeres of control and 1 Gy irradiated embryos was intact and regularly developed with intact cytoplasmic membrane and *zona pellucida* without fragments of cells in subzonal space.

Morphological defects of intensively damaged irradiated embryos were manifested by disintegrated cytoplasma of blastomeres and cytoplasmic membrane. *Zona pellucida* was intact (Figs. 4 and 5).

It was seen that the general intensity of [5-^{3}H]uridine incorporation in nucleoplasm and nucleus decreased with the increase of the irradiation dose (Table 1). The pattern of both: 1. intranuclear localization of [5-^{3}H]uridine incorporation into the irradiated embryo nucleus combined with the 2. decrease in the labelling intensity, is demonstrated in Table 1 and Figs. 2 and 3.

A high homogeneous labelling of nucleus was detected in the group of control embryos (Table 1, Fig. 1). The intensive labelling in this group of embryos was generally localized homogeneously in nucleoplasm and even more intensively in the nucleolus. Intensity of nucleus labelling decreased in the group of embryos irradiated with a dose of 1 Gy, but still remained equally localized in nucleoplasm and in nucleolus (Table 1, Fig. 2). Embryos irradiated with 2 Gy showed a very low

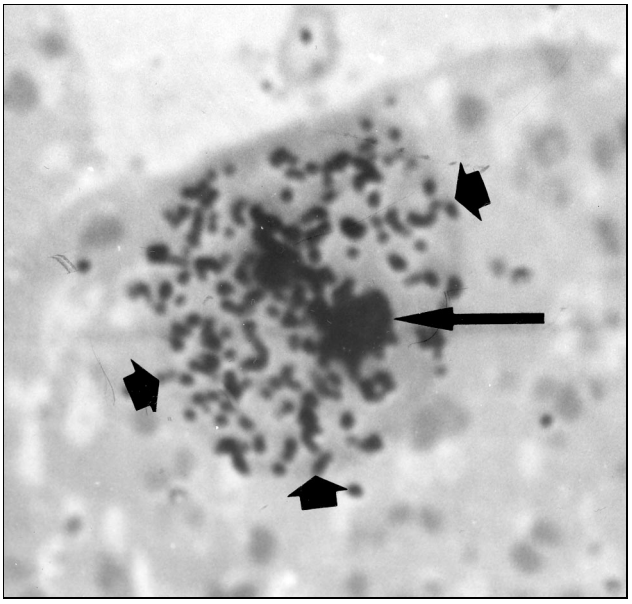

Figure 1. Light microscope autoradiography of semithin sections of 8-cell bovine embryos for the localization of [5-^{3}H]uridine incorporation into the nuclei and nucleoli (nucleus – wide arrows; nucleolus – narrow arrow). Intensive labelling of both nucleoplasma and nucleoli was typical for all 8-cell embryo nuclei without irradiation. Magnification: 1300.

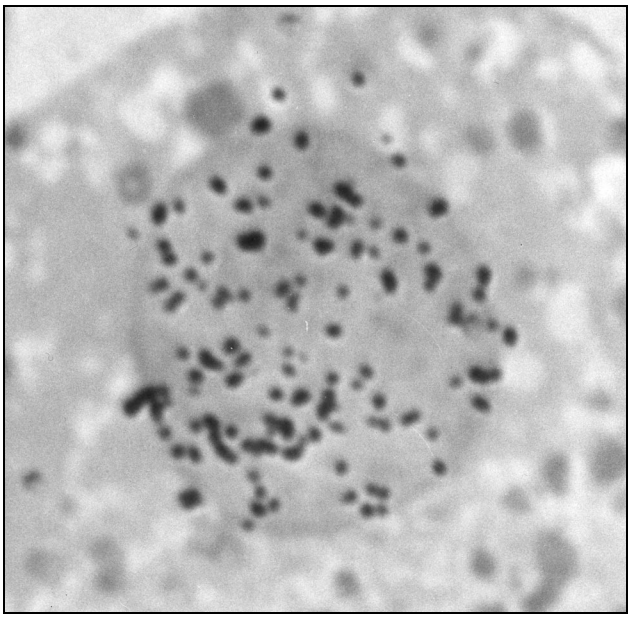

Figure 2. Intensive homogenous labelled nucleus of 8-cell embryo after irradiation with 1 Gy dose classified as category 3. Magnification: 1300.

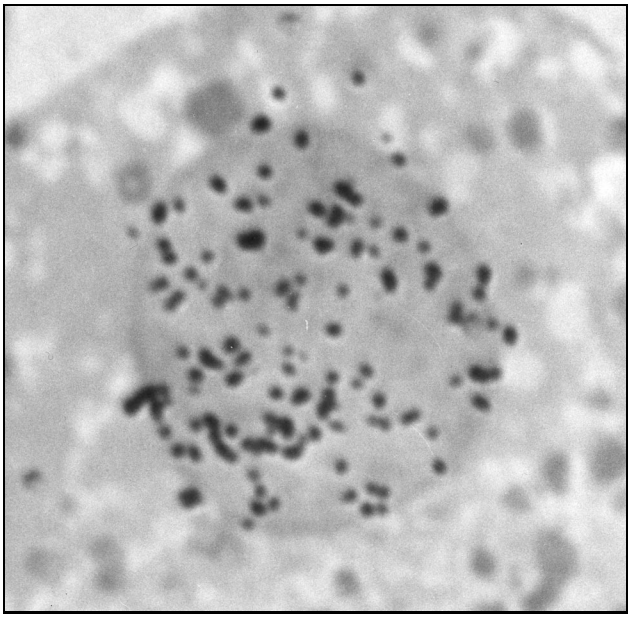

Figure 3. Low labelling intensity (category 1) of the nucleus of 16-cell embryo after irradiation with 4 Gy dose showing the low level of transcription localized mostly on the periphery of the nucleus. Magnification: 1300.

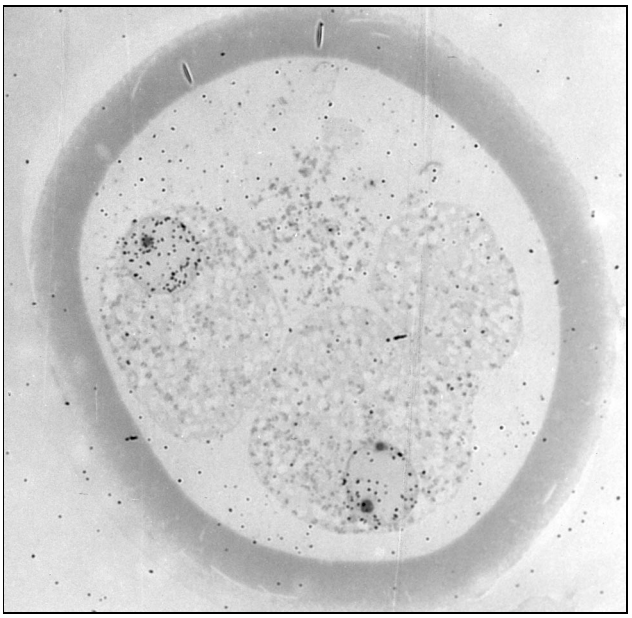

Figure 4. Light microscope autoradiography of 8-cell bovine embryos after irradiation with 2 Gy dose with an intensive disintegration of one of blastomeres. Different nuclei labelling intensity is distinct. Magnification: 500.

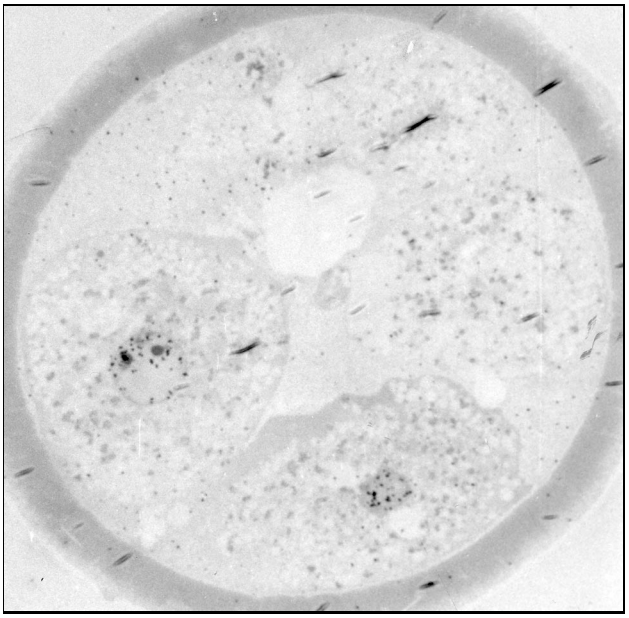

Figure 5. Low labelling intensity (category 1) of the nucleus of 8-cell bovine embryo after irradiation with 4 Gy dose. In comparison with Fig. 4 considerable disintegration and fragmentation of the majority of blastomeres are seen. Magnification: 500.

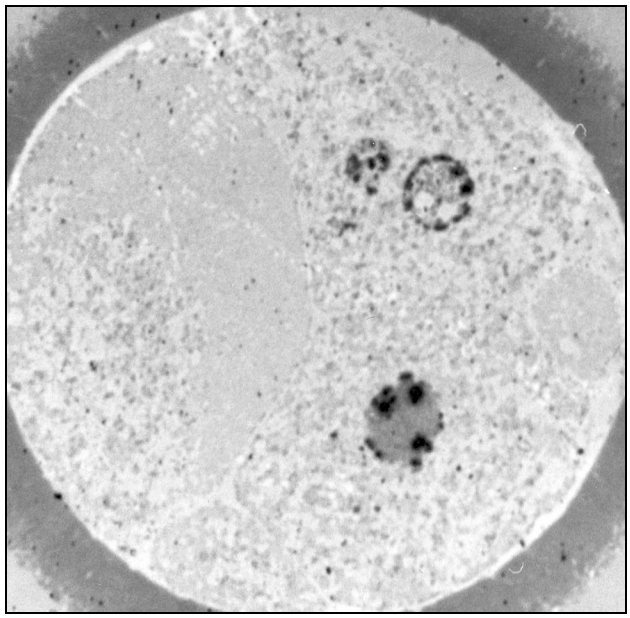

Figure 6. Light microscopy autoradiographic evidence for the localization of [5-^{3}H]uridine incorporation into nuclei of 8-cell bovine embryos after irradiation with 4 Gy dose. Total absence of the labelling is observed in nuclei with marginalization and pycnotic chromatin. Magnification: 500.

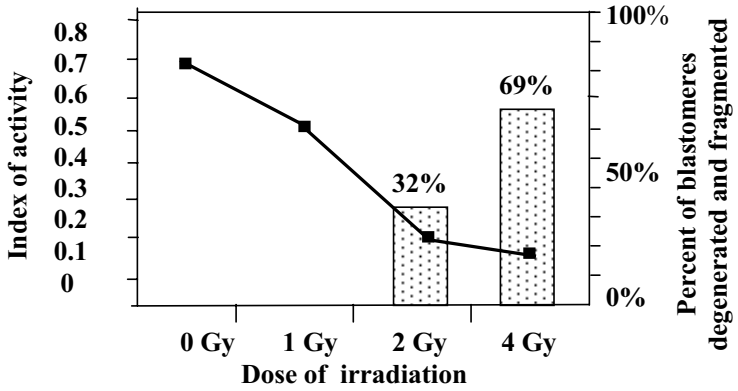

Figure 7. Broken line of decreasing intensity of the [5-^{3}H]uridine incorporation into the nuclei of 8- to 16-cell bovine embryos in each group ($n = 5$) after accute ^{60}Co irradiation with a single dose 0 Gy, 1 Gy, 2 Gy and 4 Gy; Percent of degenerated and fragmented blastomeres increasing.

labelling level localized only on the nucleoplasm periphery. In most cases, central areas of the nucleoplasm and nucleoli remained unlabelled (Table 1, Fig. 3). The 4 Gy irradiation dose resulted in a considerably greater decrease of nuclei labelling localized on the nucleoplasm periphery (Table 1, Fig. 5). Both the nucleoplasm in the nucleus center and nucleolus remained unlabelled.

Character of decreasing intensity of labelling determined by [5-^{3}H]uridine incorporation into the nuclei of blastomeres of irradiated early embryos was expressed by activity index (Table 1) calculated for each embryo group (one control and three experimental) separately. We found out that by increasing the dose of irradiation the labelling of irradiated embryo blastomere nuclei decreased. Values of activity index form the broken line of intensity of [5-^{3}H]uridine incorporation in the nuclei of blastomeres (Fig. 7).

In embryo groups irradiated with the dose of 2 Gy or 4 Gy considerable disintegration and fragmentation of cytoplasm of the blastomeres (Table 1, Figs. 6 and 7) were detected as well as various forms of chromatin segregation in nucleus, the most frequent being chromatin marginalization.

Discussion

The detection of irradiation effects in proliferating mammalian tissues is based on cell kinetics parameters and specific fine structural pathological observations (Potten 1990). At a very low dose level of irradiation the somatic cell nucleus shows impairments incompatible with the cell life already. The irradiation effects on the gamete genome differ somewhat due to its quiescent status (Rugh 1960, 1962). In that way, the irradiation impairment is detectable only after the onset of embryonic transcription (in mammals: Brent 1960, 1989; Erickson 1965; Glass and Mc Clure 1964; Russell and Montgomery 1986). A specific autoradiographic methodology for the detection of similarly delayed defects of the embryo genome due to embryos production *in vitro* from certain growth categories of antral follicles was introduced by Pavlok et al. (1993). In the present paper the applicability of this method for the detection of radiation damage in bovine embryos 16 h after irradiation was tested.

Experimental doses of irradiation used in our study (1 Gy, 2 Gy or 4 Gy) caused not only inhibition of RNA synthesis but, especially at 4 Gy dose, resulted in segregation of nuclear components. These changes were manifested mostly by formation of nucleoplasm aggregates localised often on the nuclear periphery and corresponding without doubt to generally known marginalisation of chromatin after irradiation (Peters and Stein 1964; Herich 1965; Matsudaira et al. 1967; Hugon and Borgers 1968).

Experiments with an accute irradiation in donor mice or eggs confirmed that dose of 1 Gy decreased embryo viability approximately by a half and none of mouse embryos survived the dose of 2 Gy (Glass and Mc Clure 1964). Mammalian eggs radiosensitivity differs considerably among animal species and developmental stages of the early embryos. The preimplantation stage of rat and mouse embryos is the most sensitive stage to the lethal effects of irradiation (Brent 1989). A dose of 1 Gy kills substantial numbers of mouse and rat embryos at this stage, but the survivors appear and develop normally (Russell and Montgomery 1986).

The restructurization of the nuclear morphology and the autoradiographic evaluation of the onset of major embryonic transcription in the bovine embryo

was suggested as a test for its developmental integrity (Kopečný and Niemann 1993). During these processes, the shifts of DNA-containing sites during transition from maternal to embryonic genome expression (Kopečný et al. 1989b) correlates closely with genome reactivation (Kopečný et al. 1989a; Pavlok et al. 1993). During the late 8-cell stage, namely, the embryonic DNA localized originally on nuclear periphery occupies stepwise the whole of the nucleus, probably due to the extension of the DNA molecule at the onset of transcription (Kopečný et al. 1989b). The contemporaneous association of rDNA with the nucleolus-precursor body leading to the activation of rRNA synthesis is also a morphologically well defined process which may reveal, in different experimental systems, the transcription capacity of the whole embryonic genome in cattle (Kopečný et al. 1989b; Pavlok et al. 1993). The localization of DNA in parthenogenetic bovine embryos showed further that the distribution of newly replicated DNA in the nuclei of 3- to 16-cell partenogenotes was mostly an irregular or abnormal one with a tendency to be localized on the nucleus periphery (Landa and Kopečný 1995).

In the previous works we analysed cow embryos chronically irradiated in area of Chernobyl (Pivko et al. 1997). These observations showed, that their nuclei and/or nucleolus precursor bodies (NPBs) were at the beginning of the second stadium of development (NPB_2) what was equal to the stadium of regular nucleologenesis. Differences from normometrical status, such as we described in this work had not been detected.

In the cow, the somatic histone H1 subtype is assembled onto chromatin at or near this period and this process is dependent on both embryonic transcription and DNA replication (De Sousa et al. 1998). In conclusion, the methodology of Pavlok et al. (1993) was found to detect the irradiation damage of early bovine embryo genome very sensitively, too. We suggest that a similar approach may be useful in various other situations when integrity of the embryonic genome should be tested.

Acknowledgements. The authors thank Mrs. Ľ. Dobrovodská, Mrs. A. Kováčová and Ing. P. Patráš for technical assistance.

References

Brent R. L. (1960): The indirect effect of irradiation on embryonic development II. Irradiation of the placenta. Am. J. Diseases Children **100**, 103—108

Brent R. L. (1989): The effect of embryonic and fetal exposure to x-ray, microwaves and ultrasound: couseling the pregnant and nonpregnant patient about these risks. Semin. Oncol. **16**, 347—368

De Sousa P. A, Watson A. J, Schultz G. A, Bilodeau-Goeseels S. (1998): Oogenetic and zygotic gene expression directing early bovine embryogenesis: a review. Mol. Reprod. Dev. **51**, 112—121

Erickson B. H. (1965): Symposium on atomic energy in animal science: radiation effects on gonadal development in farm animals. J. Anim. Sci. **24**, 568—583

Glass L. E., Mc Clure T. R. (1964): Survival of mouse eggs after *in vivo* or *in vitro* x-irradiation. Anat. Rec. **148**, 286

Grafenau P., Pivko J., Ovčinikov A. (1988): Superovulácia kráv Folicotropinom Spofa inj. ad usum vet. a FSH-P. Biol. Chem. Vet. **24,** 197—202 (in Slovak)

Herich R. (1965): The action of X-rays on the differentiation and development of nucleolar structures in relation to cell division. Flora (Jena) **155**, 505—510

Hrudka F. (1979): Cytochemistry of oxidoreductases in spermatozoa: the technique revisited. Andrologia **11**, 337—353

Hugon J., Borgers M. (1968): Fine structure of the nuclei of the duodenal crypt cells after X-irradiation. Am. J. Pathol. **52**, 701—723

Kaňka J., Fulka J. Jr., Peter J. (1991): Nuclear transplantation in bovine embryo: fine structure autoradiographic studies. Mol. Reprod. Dev. **29**, 110—116

Kopečný V., Fléchon J. E., Camous S., Fulka J. Jr. (1989a): Nucleologenesis and the onset of transcription in the 8-cell bovine embryo: Fine structural autoradiographic study. Mol. Reprod. Dev. **1**, 79—90

Kopečný V., Fulka J. Jr., Pivko J., Peter J. (1989b): Localization of replicated DNA – containing sites in preimplantation bovine embryo in relation to the onset of RNA synthesis. Biol. Cell **65**, 231—238

Kopečný V., Niemann H. (1993): Formation of nuclear microarchitecture in the preimplantation bovine embryo at the onset of transcription as a prerequisite for further development: Implication for biotechnology. Theriogenology **39**, 109—119

Landa V., Kopečný V. (1995): DNA synthesis and distribution in parthenogenetic bovine embryos. Theriogenology **43**, 871—881

Matsudaira H., Sekiguchi T., Nakamura C. (1967): Effects of X-irradiation on the synthesis of rapidly labelled RNA in an experimental tumour of the rat. Int. J. Radiat. Biol. **13**, 21—34

Pavlok A., Kopečný V., Lucas-Hahn A., Niemann H. (1993): Transcriptional activity and nuclear ultrastructure of 8-cell bovine embryos developed by *in vitro* maturation and fertilization of oocytes from different growth categories of antral follicles. Mol. Reprod. Dev. **35,** 233—243

Peters K., Stein G. (1964): Experimentelle Untersuchungen uber die Wirkung von Co^{60}-Gammastrahlen und Trenimon-einzeln und in Kombination auf den Kern und den Nukleolus von Krebszellen (Stamm HeLa) in der Interphase *in vitro*. Strahlentherapie **125**, 258—272

Pivko J., Baran V., Grafenau P., Kopečný V., Pelechatyj N. S., Bondarčuk V. N., Kožuch A. J., Kovalčik L. M. (1997): A study of the dynamical ultramicromorphology of differentiating cow early embryo nucleoli in embryos collected in the area of chronic irradiation near Tchernobyl. Vet. Med. (Prague) **2**, 33—38

Potten C. S. (1990): A comprehensive study of the radiobiological response of the murine (BDF_1) small intestine. Int. J. Radiat. Biol. **58**, 925—973

Rugh R. (1960): Mechanisms in Radiobiology (Eds. M. Errera and A. Forsbers), Vol 2., New York, Academic Press

Rugh R. (1962): Low Levels of X-Irradiation and the Early Mammalian Embryo. Am. J. Roentgenol., Radium Therapy Nucl. Med. **87**, 559—566

Russell L. B., Montgomery C. S. (1986): Radiation-sensitivity differences within cell-division cycles during mouse cleavage. Int. J. Radiat. Biol. **10**, 151—164

Stringfellow D. A., Seidel S. M. (1990): Manual of the International Embryo Transfer Society. Second edition, IETS, Champaing, IL, U.S.A.

Telford N. A., Watson, A. J., Schultz G. A. (1990): Transition from maternal to embryonic control in early mammalian development: A comparison of several species. Mol. Reprod. Dev. **26**, 90—100

Final version accepted: April 12, 2001