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Effect of ^{60}Co gamma radiation on *Biomphalaria glabrata* (Mollusca, Gastropoda) embryos: mortality, malformation and hatching

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Abstract

A study was carried out on the radiosensitivity of *Biomphalaria glabrata* embryos submitted to doses of 5, 10, 15, 20 and 25 Gy of ^{60}Co during the cleavage, blastula, gastrula, young trochophore and trochophore stages. Mortality, malformation and hatching were the parameters used to evaluate the damage induced by ionizing radiation. Estimated LD_{50} values (15 days) showed that the cleavage stage (4.3 Gy) was approximately four times more radiosensitive than the trochophore stage (17.0 Gy). Susceptibility to malformation induction was higher in the blastula, gastrula and young trochophore stages. Several types of morphogenetic malformations were observed, such as head malformations, exogastrulas, shell malformations, and embryos with everted stomodeum, with nonspecific malformations being the most frequent. The types of malformation induced by radiation probably are not radiation-specific and do not depend on the dose applied. The dose of 15 Gy was sufficient to greatly reduce the number of hatching snails regardless of the embryonic stage irradiated. We conclude that the effect of ^{60}Co gamma radiation on *B. glabrata* embryos presented a specific pattern.

Key words
- *Biomphalaria glabrata* embryos
- Ionizing radiation
- Survival curves
- LD_{50} values
- Malformations
- Hatching
- Schistosomiasis

Introduction

The study of the effect of ionizing radiation on embryo development has been attracting increasing interest not only from the viewpoint of experimental embryology but also for the evaluation of radiation damage during the initial process of ontogenetic development. Knowledge of the effects of ionizing radiation on the human embryo or fetus is limited and comes fundamentally from studies of A-bomb survivors and of persons exposed to internal or external sources during radiologic, therapeutic or diagnostic procedures (1). Studies using helminths (2), molluscs (3), insects (4), fish (5), amphibians (6) and mammals (7,8) have shown that the changes induced by radiation are qualitatively similar in different species when the animals are irradiated at equivalent stages of development. However, the mechanisms leading to morphogenetic anomalies are not well understood.

Egg masses of the snail *Biomphalaria*

glabrata are suitable for the study of the effect of radiation on embryonic development since this organism is easy to maintain and manipulate and has a relatively short embryonic period (approximately one week), which allows monitoring each embryonic stage *in vivo*. The embryology of *B. glabrata* has been analyzed in detail by Camey and Verdonk (9). In addition, investigation of this snail species is of special interest since it is a vector of schistosomiasis, an endemic disease that affects more than 200 million people, mainly in South America, Africa and the Middle East (10).

B. glabrata has been extensively studied due to its medical and social relevance, but little information is available on the effect of ionizing radiation on *B. glabrata* embryos. Perlowagora-Szumlewicz and Berry (11) and Perlowagora-Szumlewicz (12) explored the possibility of utilizing ionizing radiation as a tool for biological control of *B. glabrata*. Kawano (13) investigated the effects of caffeine as a synergistic agent of gamma radiation at a dose of 10 Gy on the embryonic development of an albino mutant (SL) *B. glabrata*. Narang (14) and Narang and Narang (15) analyzed the cytogenetic effects of X-ray in the ootestis of irradiated snails and in F_1 embryos. Okazaki and Kawano (16) investigated the association between radioinduced chromosome aberrations and the morphogenetic effects of ^{60}Co gamma radiation on *B. glabrata* embryos.

In the present study we investigated the radiosensitivity of *B. glabrata* embryos at different stages of development using mortality, malformation and hatching as criteria for the evaluation of ionizing radiation damage.

Table 1 - Effect of ^{60}Co gamma radiation on *Biomphalaria glabrata* embryos at several stages of development, on the 15th day after irradiation.

Embryonic stage	Dose (Gy)	Number of egg masses	Number of embryos	Dead embryos (%)	Surviving embryos (%)		
					Malformed	Hatched	Unhatched "normal"
Cleavage	0	28	557	8.40	0.20	90.40	1.10
	5	28	693	55.80	3.60	34.60	5.90
	10	29	701	80.40	2.80	13.50	3.30
	15	29	596	93.50	0.50	5.20	0.80
Blastula	0	44	1154	6.50	0.20	91.10	2.38
	5	41	1032	24.70	11.70	48.60	15.00
	10	41	1130	53.60	17.80	14.40	14.20
	15	41	918	76.70	15.40	5.50	2.40
Gastrula	0	40	994	3.20	0.00	96.80	0.00
	5	41	1085	11.70	2.70	79.40	6.20
	10	41	1060	40.90	9.60	16.00	33.50
	15	41	1019	85.60	10.60	0.40	3.40
Young trochophore	0	35	773	4.90	0.30	92.60	2.30
	5	38	766	8.10	0.40	88.50	3.00
	10	41	858	40.70	5.50	26.10	27.70
	15	38	1034	67.90	10.80	0.50	20.80
	20	27	895	91.70	8.00	0.00	0.20
Trochophore	0	42	953	3.10	0.00	96.50	0.40
	5	42	931	3.90	0.00	95.40	0.60
	10	42	908	7.80	0.70	66.40	25.20
	15	42	1111	28.40	7.50	9.10	55.00
	20	24	770	64.00	8.10	1.90	26.00
	25	25	840	95.00	1.80	3.20	0.00

Material and Methods

Egg masses from wild-type *Biomphalaria glabrata* (Say, 1818) (Mollusca, Gastropoda) originally collected in Belo Horizonte, MG, Brazil and reared under laboratory conditions were used. The following embryonic stages of *B. glabrata* were selected: a) first cleavages, including the first, second and third cleavages (0 to 3.5 h of age), b) blastula (6 to 15 h), c) gastrula (24 to 30 h), d) young trochophore (48 to 54 h) and e) trochophore (72 to 78 h after the first cleavage) (17). Embryo stages were identified by the criteria of Camey and Verdonk (9).

For each embryonic stage, egg masses were divided at random into 4 or 6 groups, each containing approximately 100-200 embryos. Each group was irradiated separately with single doses of 5, 10, 15, 20 and 25 Gy gamma radiation (136 Gy/h) using a ^{60}Co Gamma-Cell 220 source (Irradiation Unit of the Canadian Atomic Energy Commission Ltd.). One group from each embryonic stage was used as unirradiated control. On the basis of preliminary experiments, the doses from 5 to 25 Gy were selected as the lethality range from the sublethal dose to the dose that induced approximately 90% mortality. Because of the high mortality rate observed in the early stages submitted to 15 Gy, higher doses were applied only to the late stages such as young trochophore (20 Gy) and trochophore (20 and 25 Gy).

After irradiation, the egg masses were kept at 25 ± 1°C throughout the experimental period to maintain the normal rhythm of embryonic development. Egg masses were observed daily with a Zeiss stereoscopic microscope up to the 15th day after irradiation. This time interval was considered to be adequate to determine the behavior of the embryos submitted to irradiation since the normal embryonic period is relatively short, i.e., approximately one week, at 25°C. Five replicates were performed for each embryonic stage irradiated and control embryos.

The mortality data were fitted to the $Y = \exp(-(\alpha D - \beta D^2))$ model by the least squares method using the BMP3R subroutine. Y represents the survival observed after dose D, and α and β are the parameters of the model.

Malformed embryos were identified using the criteria of Verdonk (18) and Geilenkirchen (19). Embryos showing a size reduction or developmental delay were considered "normal" as long as no identifiable malformation was detected *in vivo*.

For *in toto* embryonic analysis, the material was prepared according to the technique described by Verdonk (18), which basically consists of the following steps: decapsulated embryos were fixed with 0.75% $AgNO_3$, dehydrated in an alcohol series, cleared with xylene and mounted with Permount between a slide and a coverslip. Embryos were drawn using a Zeiss camera lucida.

Results

Table 1 shows the mortality, malformation and hatching rates of *B. glabrata* embryos at several stages of development obtained on the 15th day after irradiation.

Mortality

Figure 1 presents the adjusted survival curves for *B. glabrata* embryos after irradiation during the various phases of development. An initial "shoulder" within the lower

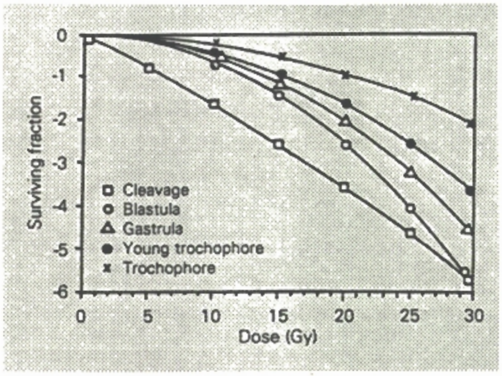

Figure 1 - Adjusted survival curve of *B. glabrata* embryos irradiated at several stages of embryonic development.

Table 2 - Estimates for the dose-response model for *Biomphalaria glabrata* survival obtained on the 15th day, for each stage of embryonic development.

Stage	Parameter	Estimate	Standard deviation	Residual sum of squares
Cleavage	α	0.155	0.048	0.003
	ß	0.001	0.005	
Blastula	α	0.036	0.018	0.002
	ß	0.004	0.002	
Gastrula	α	0		0.005
	ß	0.006	0.001	
Young trochophore	α	0		0.001
	ß	0.005	0.001	
Trochophore	α	0		0.001
	ß	0.002	0.001	

Table 3 - Estimated LD_{50} values (15th day) for each stage of *Biomphalaria glabrata* embryonic development.

Embryonic stage	LD_{50} (15 days) (Gy)
Cleavage	4.3
Blastula	9.3
Gastrula	10.3
Young trochophore	11.5
Trochophore	17.0

dose range was observed which became more marked as development progressed. The curves decreased exponentially as a function of radiation dose at all embryonic stages, being more pronounced during the earlier stages.

The estimated values of the parameters of the model with their respective standard deviations and residual sums of squares for each embryonic stage are presented in Table 2, with adequate fitting of the data to the proposed model. LD_{50} estimates of the values for the 15th day after irradiation for each embryonic stage are presented in Table 3, showing that adjusted LD_{50} values increase with embryonic stage.

Malformation

Figure 2 represents the frequencies of malformed embryos obtained throughout the experiment as a function of radiation dose. Table 4 shows the modes of malformed embryo frequencies obtained with various radiation doses during the different developmental stages. The mode of malformed embryo frequencies increased with progressive radiation dose and with decreasing embryonic stages for the same dose. However, in the cleavage stage there was a significant decrease in the modes of malformed embryonic frequencies for increasing radiation dose ($Y = 0.53-0.02X$, $b = 0$, $R = 0$, $F_{1,13} = 22.89$, $P<0.05$).

The morphogenetic malformations were predominantly of the nonspecific type (Figure 3) in all embryonic stages and at all doses investigated. However, exogastrulas and some head malformations such as monophthalmia and eye reduplication type (Figure 4) were obtained in eggs irradiated in the earliest stages, while stomodeum eversion and shell malformations were observed after irradiation of the more advanced stages such as young trochophore and trochophore.

Hatching

Figure 5 shows the effect of gamma radiation applied during the various developmental stages on *B. glabrata* embryo hatching. The hatching rate decreased considerably with increasing radiation dose at all embryonic stages. At 15 Gy, the percentages of hatched eggs were greatly reduced at all embryonic stages investigated.

Control embryos from the same egg mass seldom hatched simultaneously. At 25°C, the embryos hatched between the 6th and 9th day after first cleavage. In sublethally irradiated egg masses, hatching occurred with a difference in phase of 1 to as many as 4 days in relation to the first day of hatching of control embryos. The irradiated embryos that hatched seldom presented visible malformations. Some embryos did not hatch until the 15th day after exposure (Table 1). These embryos were apparently "normal" and undistinguishable from control embryos.

The percentage of unhatched snails was particularly higher in egg masses irradiated with 15 Gy at the trochophore stage (about 55%).

Discussion

B. glabrata embryos showed a relatively high susceptibility to the action of gamma radiation, the intensity of damage depending on dose and embryonic stage at the time of irradiation. The cleavage stage was the most radiosensitive at all doses analyzed, with more than 90% of all individuals dying at the 15 Gy dose. Similarly, the blastula, gastrula and young trochophore stages were sensitive to treatment, though the sensitivity for survival was not as marked as that observed in the cleavage stage. The least affected phase was the trochophore at the 5 to 15 Gy dose, although at higher doses there was a considerable dose-dependent increase in mortality. Estimated LD_{50} (15 days) showed that the cleavage stage was approximately four times more radiosensitive than the trochophore stage.

The survival curves were similar to those

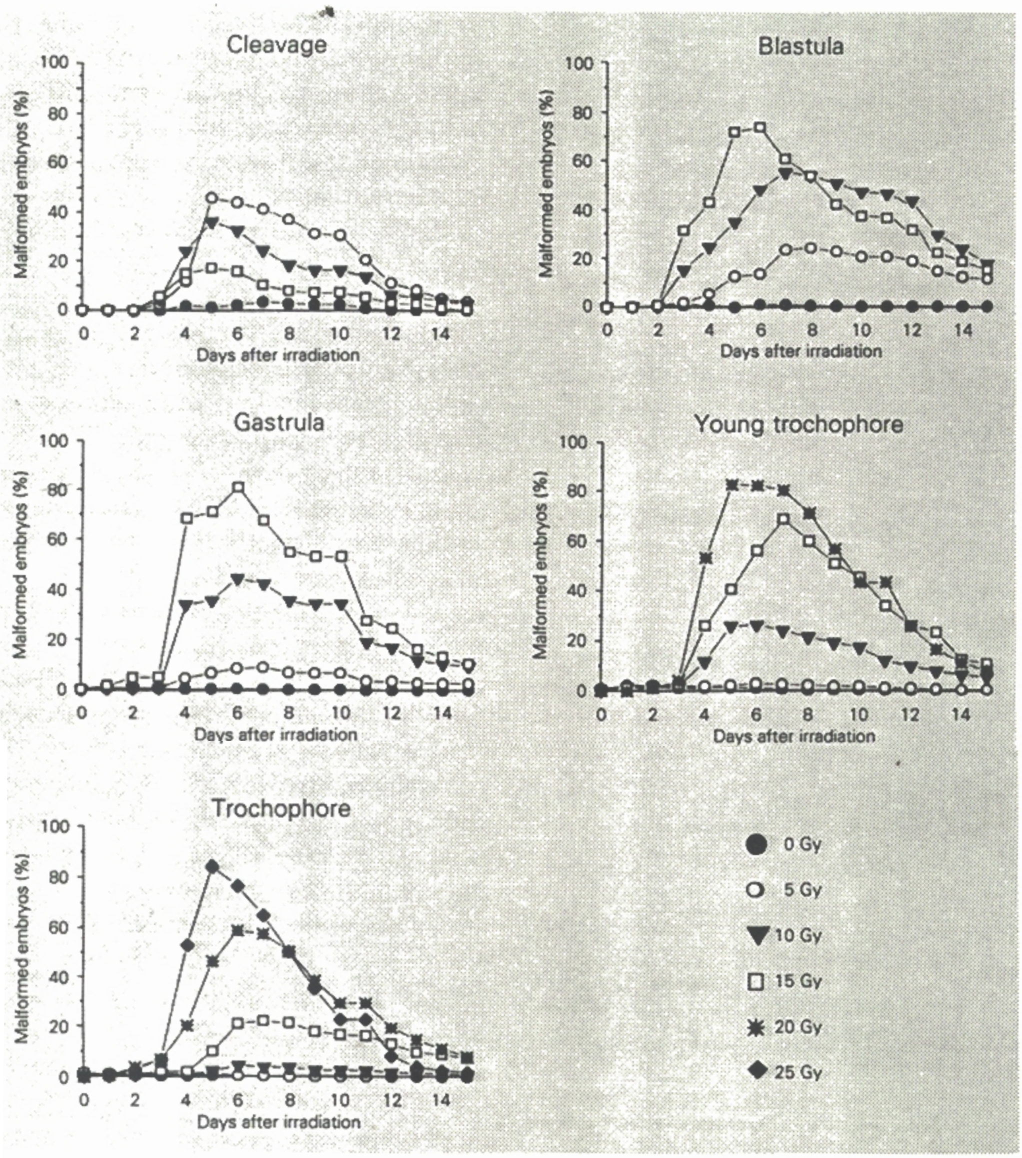

Figure 2 - Number of malformed *B. glabrata* embryo curves obtained after ^{60}Co gamma irradiation at different developmental stages.

obtained for mammalian cells exposed to low LET radiation, which fit better the theoretical multi-target curve (20). On the basis of this model, the shoulder region of the survival curve may indicate an accumulation of sublethal damage. The probability of more embryo cells being affected may increase with growing radiation doses, resulting in death of the organism. These observations suggest a cumulative damage of ionizing radiation.

The presence of a shoulder in the survival curve may also be due to a repair process (20), and the increased lethality observed with growing radiation doses may be due to insufficient repair. In the early cleavage stage there was practically no shoulder and the higher sensitivity for survival as a function of dose was more marked than in the more advanced stages.

Proliferative cells are known to be highly radiosensitive and the susceptibility varies with cell cycle phase, being higher during mitosis than during interphase. So, the cell cycle phase at the irradiation time may be an important factor in the determination of embryo sensitivity to ionizing radiation.

In the earlier stages of development, for example during the 2-blastomere phase, the death of one cell is critical for the development of an organism since this cell represents the precursor of half of the cells of an embryo. At this stage, an all-or-nothing effect seems to operate at the cell level, depending on the susceptibility of the cell cycle phase at the time of irradiation.

As the embryo develops there is a progressive increase in cell differentiation with tissue and organ specialization, which coincides with embryo radioresistance. In the trochophore stage, the least affected by radiation, most of the rudimentary organs of the adult are almost defined (9). As early as 1906, Bergonié and Tribondeau (21) observed that the radiosensitivity of mammalian cells or tissues was directly proportional to their proliferation rate and inversely pro-

Table 4 - Modes of malformed embryo frequencies obtained with different radiation doses at the different developmental stages.

Embryonic stage	Dose (Gy)	Number of days after irradiation	Malformed embryos (%)
Cleavage	0	7	3.5
	5	5	45.9
	10	5	36.3
	15	5	17.4
Blastula	0	6	1.1
	5	8	24.3
	10	7	55.5
	15	6	73.8
Gastrula	0	6	0.7
	5	7	9.2
	10	6	44.6
	15	6	81.2
Young trochophore	0	1	2.3
	5	6	3.1
	10	6	26.3
	15	7	68.7
	20	5	82.7
Trochophore	0	3 and 4	1.2
	5	1	1.2
	10	6	4.6
	15	7	22.4
	20	6	59.0
	25	5	84.3

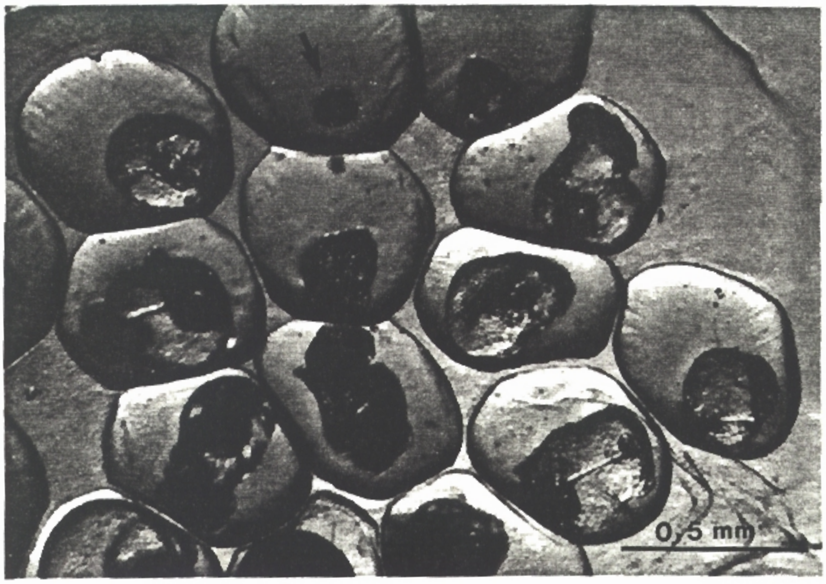

Figure 3 - Egg mass irradiated with 10 Gy at the blastula stage, on the 6th day after treatment, showing several nonspecific malformed embryos. The arrow shows a dead embryo. X48.

portional to their degree of differentiation. Even though some exceptions to this rule are known to exist, these observations have been confirmed in most biological systems.

The malformed embryo curves obtained for *B. glabrata* eggs irradiated at different developmental stages showed that the frequency of malformed embryos increased rapidly with increasing radiation doses, reaching a maximum between the 5th and 8th day after irradiation and later decreasing due to gradual embryo death. The malformations presented by the embryos were probably so severe that most of them were unable to survive and died before hatching. On the other hand, the survivors that reached hatch-

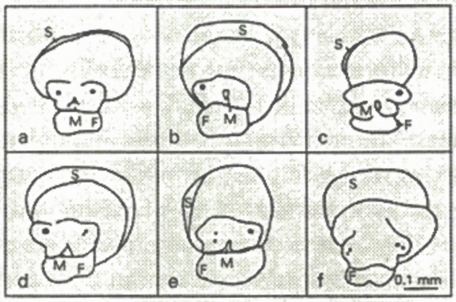

Figure 4 - Outline drawings of head malformations found in *B. glabrata* embryos irradiated at the blastula stage with 10 Gy of gamma radiation and fixed on the 5th day after exposure, at the veliger stage. a) Normal embryo, b) right monophthalmia, c) left monophthalmia, d) left reduplication, e) right reduplication, f) right and left reduplication. M = Mouth, S = shell, F = foot.

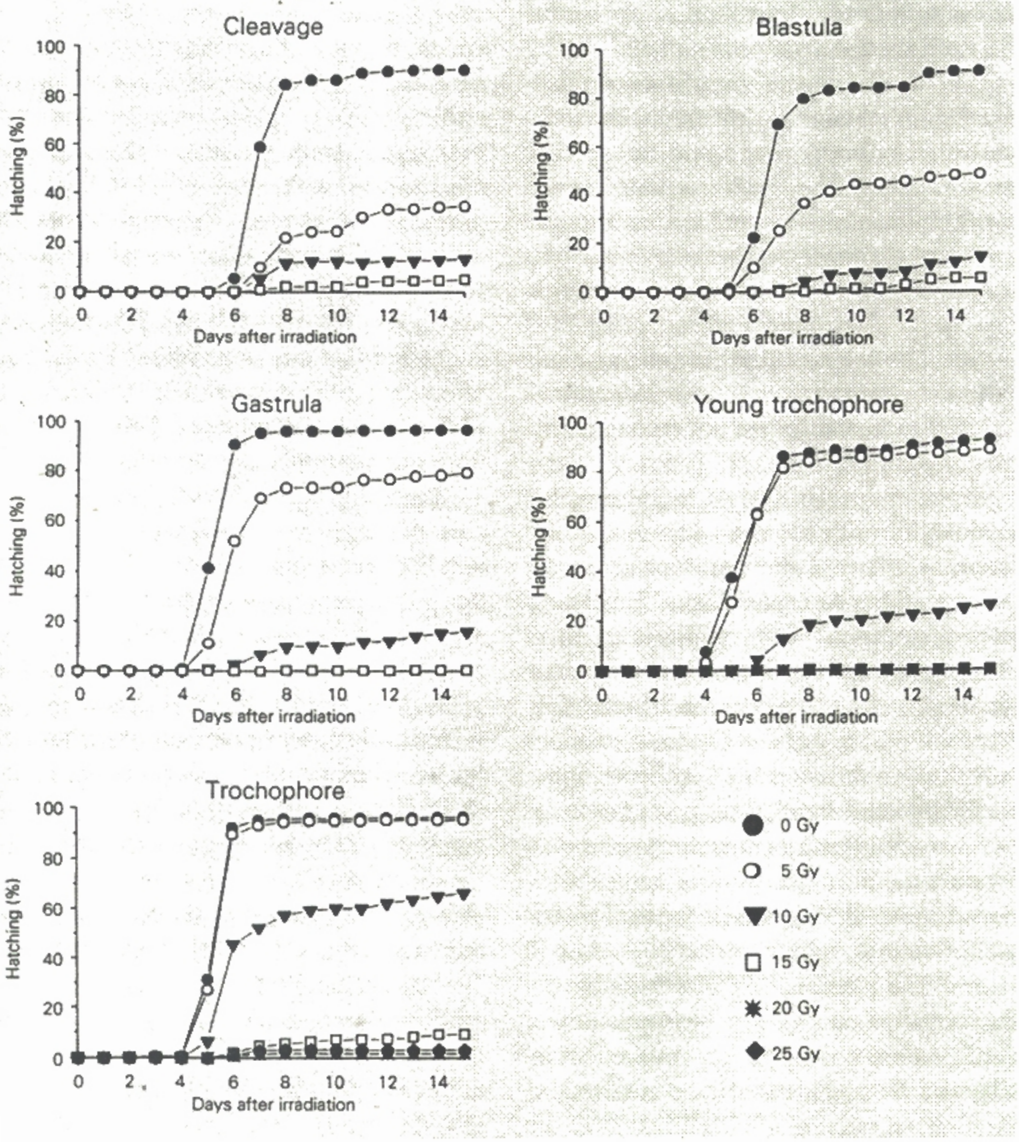

Figure 5 - Cumulative hatching curves of *B. glabrata* eggs submitted to ionizing radiation at several developmental stages.

ing seldom showed visible malformations.

In the cleavage stage, however, the rate of malformed embryos decreased with increasing radiation dose. A significant decrease in malformed embryo frequency modes was due to the progressive increase in embryo mortality with increasing radiation doses, as a direct consequence of the lethal action of radiation during their early developmental stage.

The more evolved the embryo, the higher the dose needed to produce a quantitatively similar effect in relation to earlier embryos. The modes tended to follow the increase in radiation dose: the higher the dose, the shorter the time needed to detect its effect.

The same types of morphogenetic malformations obtained with irradiation (exogastrula, hydropia, head and nonspecific malformations, shell malformation, everted stomodeum) are also produced in mollusc embryos by different agents such as heat shock (22), UV radiation (23), centrifugation (24), blastomere removal (25), LiCl (18) and caffeine (26). These observations show that the morphogenetic malformations induced by radiation are not radiation specific.

Apparently, the type of morphogenetic anomaly resulting from radiation of *B. glabrata* embryos did not depend on the dose applied. Nonspecific malformations were the predominant type detected at all doses analyzed. However, their frequency was clearly dose dependent, as observed by Verdonk (18) in eggs of *Limnaea stagnalis* submitted to different LiCl concentrations.

On the other hand, there was an association between types of morphogenetic malformation and stages of development submitted to irradiation except for nonspecific malformations, which were observed at all stages. The precursor cells responsible for the formation of the entire head structure of the *B. glabrata* embryo, except the mouth, originate from the cellular descendants of the first micromere quartet (1a-1d) at the third cleavage stage (9). Several investigators have suggested that the inductive process for gastrulation probably occurs also in the first cleavage stages (27). Thus, the action of physical or chemical agents during these developmental stages may induce a deviation in head pattern or a suppression of gastrulation, with the consequent occurrence of anomalies such as head malformation or exogastrula.

The head malformations of the monophthalmia and eye reduplication types detected in the present study suggest a possible interference of ionizing radiation with the chronology of division of the cephalic plates (future regions of eye and tentacles) which were probably in a phase of intense proliferation. Since the apical plate and the cerebral vesicle were probably already formed (9), the head malformations of the cyclocephalic series were not observed. Structures such as the stomodeum and shell gland are formed at more advanced stages (young trochophore and trochophore). Thus, it was suggested that ionizing radiation somehow affects the cells during the morphogenesis of these organs, resulting in shell malformations and stomodeum evagination.

These data lead us to assume that, in the case of nonspecific malformations, several cell lines were probably affected by radiation. Thus, embryos exposed to radiation may present irregular development, with more affected parts developing less and other areas growing and developing in a normal manner. This does not exclude other factors, but the morphogenetic significance of the chronology of cell division deserves special attention, since this event is a prerequisite for the establishment of a normal relationship between cells and tissues, with great relevance for the developing organism (28).

The hatching rate of irradiated *B. glabrata* embryos decreased as a function of radiation dose and, at the same dose, increased with age of the irradiated embryo. The least affected stage was the trochophore, with no difference in hatching rate between eggs irradiated with 5 Gy (95.4%) and controls (96.5%). However, a 15-Gy dose was sufficient to reduce considerably the hatching rate, regardless of the embryonic stage irradiated. A decrease in hatching rate proportional to increasing radiation dose has also been observed in eggs of *Pomacea haustrum* (Prosobranchia, Pilidae) (29) and of *Physa acuta* (Gastropoda) (30).

Perlowagora-Szumlewicz and Berry (11) and Perlowagora-Szumlewicz (12), however, noted that higher doses were needed to prevent hatching of hybrid *Australorbis glabratus*. The hatching rate of 6-8-day old embryos irradiated with 20 Gy of X-rays was similar to controls, and 9-10-day old embryos (veliger) resist up to 400 Gy, with a 68% hatching rate, dying thereafter.

The difference between the results cited above and ours may be due to distinct radiation doses and schedules used or a greater radioresistance of the strain employed by these investigators. Even within the same species, different strains may show different degrees of radiosensitivity (31).

The delay in hatching observed in sublethally irradiated *B. glabrata* embryos may have been partly due to the slower development of the embryos, or to slow recovery from the sublethal damage induced by radiation. The marked decrease in hatching rate with increasing radiation doses, especially when early developmental stages were irradiated, was largely due to the high embryo mortality caused either by the direct lethal action of radiation or by the death of malformed embryos. However, in the more advanced stage like trochophore, this decrease in hatching rate was caused by inhibition of the hatching process alone (unhatched "normal" embryos). Later observations showed that these embryos died within the egg capsule between the 20th and the 30th day after irradiation.

The hatching of helminth (32), amphibian and fish (33,34) eggs occurs by the action of hatching enzymes that digest the egg membranes. Identified hatching enzymes include a kitinase and an esterase in *Ascaris lumbricoides* (32) and a tryptic proteinase in *Oryzias latipes* (35). The enzymes are secreted by cells of the hatching gland, which, according to Hyodo-Taguchi et al. (36), may be the critical tissue for hatching. Shimada et al. (37) correlated the different hatching rates observed in *O. latipes* embryos, irradiated during the various embryonic stages, with the developmental stage of the hatching gland. These investigators suggested that radiation at early developmental stages may affect the multiplication and differentiation of hatching gland cells and, at more advanced stages, may affect the synthesis or secretion of hatching enzymes, thus inhibiting egg hatching.

In the eggs of certain gastropods, hatching appears to occur mechanically (34). Rey (38) and Geraerts and Joosse (39) reported that, in Basommatophora, hatching occurs through the action of the radula (the chewing apparatus of the snail) which, together with the movement of the snail itself within the egg, breaks the egg membrane at the time of hatching. The radula originates from micromeres of the 2nd (4th egg cleavage) and 3rd quartets (blastula) and differentiates at the trochophore stage (40). However, the planorbids, in addition to the radula, seem to utilize an enzyme that favors hatching by liquefying a gelatinous substance covering the embryos or even by acting on the membrane of the egg capsule (41).

Together with the above information, our results suggest that the marked decrease in hatching rate observed in embryos sublethally irradiated at more advanced stages may be due to a blockage of hatching brought about by radiation-induced damage in the formation of the radula or in the synthesis of a specific hatching enzyme.

In conclusion, our results show that, in irradiated *B. glabrata* eggs, the mortality caused by the direct lethal action of radiation was the most pronounced event in the earliest developmental stage, whereas the hatching inhibition was found in the more advanced stage, such as the trochophore. The higher incidence of morphogenetic malformations was significant in the blastula, gastrula and young trochophore stages, coinciding with the intense cell proliferation, morphogenetic movement and major organogenesis periods. These data may contribute to a better understanding of the biology of these snails, providing insights for the environmental control of schistosomiasis.

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