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Comparison of biological effects between continuous and intermittent exposure to GSM-900-MHz mobile phone radiation: Detection of apoptotic cell-death features

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ABSTRACT

In the present study we used a 6-min daily exposure of dipteran flies, *Drosophila melanogaster*, to GSM-900 MHz (Global System for Mobile Telecommunications) mobile phone electromagnetic radiation (EMR), to compare the effects between the continuous and four different intermittent exposures of 6 min total duration, and also to test whether intermittent exposure provides any cumulative effects on the insect's reproductive capacity as well as on the induction of apoptotic cell death. According to our previous experiments, a 6-min continuous exposure per day for 5 days to GSM-900 MHz and DCS-1800 MHz (Digital Cellular System) mobile phone radiation, brought about a large decrease in the insect's reproductive capacity, as defined by the number of F_1 pupae. This decrease was found to be non-thermal and correlated with an increased percentage of induced fragmented DNA in the egg chambers' cells at early- and mid-oogenesis. In the present experiments we show that intermittent exposure also decreases the reproductive capacity and alters the actin-cytoskeleton network of the egg chambers, another known aspect of cell death that was not investigated in previous experiments, and that the effect is also due to DNA fragmentation. Intermittent exposures with 10-min intervals between exposure sessions proved to be almost equally effective as continuous exposure of the same total duration, whereas longer intervals between the exposures seemed to allow the organism the time required to recover and partly overcome the above-mentioned effects of the GSM exposure.

1. Introduction

Mobile phones are widely used and during the past few years we have witnessed a continuous accumulation of information concerning the possible health hazards from exposure to the electromagnetic radiation associated with mobile telephony. Many studies have been performed during the last years investigating a variety of biological effects, including epidemiological/clinical studies in humans and experimental work on rodents, flies, or cell cultures.

Assessing the possible link between exposure to electromagnetic fields (EMF) and genotoxic effects, a number of studies have reported DNA or cell damage, such as DNA breaks, cell malformations, cell death, changes in chromatin conformation and micronucleus formation in different cell types or organisms [1–9]. In other studies, no genotoxic effects of exposure to EMF were observed [10–22].

Mobile phone radiation has been also found able to cause broad changes in gene and protein expression in certain cell types [17,23–25]. According to other studies, there is evidence that molecular damage caused by Radio Frequency (RF)-microwave fields activates the stress response of the cells, which then acts as a natural defense mechanism against this kind of stimuli. Such a response may be detected through an increase in reactive oxygen species (ROS) or as increased levels of stress proteins [26–28]. A number of *in vivo* studies have also been carried out on possible effects of EMF at the level of the whole organism [28–33]. On the other hand, other authors reported no change in gene expression in *in vitro* studies [34], no change in mutation frequencies and no behavioural or morphological effects in *in vivo* studies after exposure to RF fields [35,36].

With regards to the comparative biological effects between intermittent and continuous exposure, Diem et al. [6], reported that intermittent exposure to RF with 10-min intervals between doses (1800 MHz, SAR = 2 W/Kg), had stronger effects than a single, continuous exposure of the same total duration on human diploid fibroblasts and on rat granulosa cells, producing higher levels of DNA single and double strand breaks. In earlier experiments, the same group compared the effects of an extremely low

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frequency (ELF) field (50 Hz, 10 G), on human diploid fibroblasts using different intervals between exposures, demonstrating the crucial role of the combination between the duration of exposure and its intermittence. They found that the type of intermittent exposure producing the highest degree of DNA damage was the one with 5-min exposures and 10-min intermittence between them, while longer intervals between exposures reduced the extent of the effects [37]. Attempts were made to reproduce these experiments in other laboratories with the same RF or ELF exposure conditions and the same cells, but the results were not confirmed [19,20]. Also, according to other studies, intermittent RF exposure did not affect mutation frequencies in the *lacZ* gene in pregnant mice [35] or gene expression in human-derived cell lines [34].

Based on the current data, it appears that the duration of the exposure, the exposure pattern, the intensity of radiation, as well as the type and sensitivity of the biological system used, all play an important role in the observed biological effects of EMF [24,25,38–42].

The insect *Drosophila melanogaster* has been proven to be extremely useful in studies of EMF effects, due to the wealth of available genetic information in combination with the thoroughly studied developmental biology of oogenesis.

The *Drosophila* ovary is divided into discrete units called ovarioles. Each ovary consists of 18–20 discrete tubular ovarioles, each containing 6–7 independent, developmentally ordered follicles or egg-chambers. Each egg chamber consists of 1 oocyte, 15 nurse cells and approximately 1.000 follicle cells surrounding the oocyte [43]. These cell types are formed in the germarium at the anterior end of the ovary. Egg chambers leave the germarium as stage-1 egg and the entire process of oogenesis involves 14 stages [44] where a stage-14 oocyte is mature to be fertilized and oviposited. The different stages have been fully characterized using morphological, biochemical and molecular criteria [45,46]. Egg chambers develop sequentially within the ovarioles, and thus a single ovary contains egg chambers of all stages of oogenesis [47].

Programmed cell death in the *Drosophila* ovary has been extensively studied and occurs in response to both developmental and environmental stimuli, following two different patterns, playing an integral role in the normal development of every oocyte [48–50]. According to the first developmentally regulated and asynchronous pattern, physiological apoptotic cell death commences at stage 11 in the nurse cells of every normally developing follicle, with the rearrangement of the peri-nuclear actin-cytoskeleton. At stage 12, chromatin condensation proceeds and ends with the DNA fragmentation of the nurse-cell nuclei at stage 13. The cell remnants are engulfed by the neighbouring follicle cells that also die physiologically at stage 14 [51–53]. The second pattern, which is sporadically observed at stages 7–8 and in region 2 in the germarium, is induced in response to different stress stimuli and it is marked simultaneously by the condensed, fragmented nurse-cell and follicle-cell nuclei and the disorganized actin network of the abnormal follicles. It is now clear that the stages 7–8 and the region 2 within the germarium are the two checkpoints that provide a protective mechanism in the process of oogenesis: they remove defective egg chambers that are unable to develop into fertile eggs, thus preventing the waste of precious nutrients [51,54].

In our previous experiments it was shown that a short daily exposure of newly emerged adult flies of *D. melanogaster* to mobile phone radiation, decreases significantly and non-thermally their reproductive capacity, as defined by the number of F_1 pupae [32,33]. We have also shown that in response to the stress from exposure of the insects to this radiation, fragmented DNA was detected in all the developmental stages of early- and mid-oogenesis (*i.e.* from the germarium stage up to stage 10) and in all three different types of egg-chamber cell. The above-mentioned checkpoints, germarium and stage 7–8, were shown to be the most sensitive developmental stages also to this kind of electromagnetic insult [9]. The observed DNA fragmentation and the induced cell death explain the above-mentioned decrease in the reproductive capacity [32,33]. Other recent findings of our group have indicated that the effect on the reproductive capacity increases almost linearly with increasing daily exposure duration from 1 to 21 min to GSM-900 and 1800-MHz EMF and that the effect is non-thermal [38].

The aim of the present study was to compare the influence of different types of intermittent exposure with different intervals, with the effects of continuous exposure of the same total duration of GSM radiation on the reproductive capacity of *D. melanogaster*. Consequently, the study aimed to examine whether the various intervals of intermittent exposures would indicate the possible existence of repair mechanisms operating in the biological system tested. Furthermore, our aim was to test – in addition to the confirmation of DNA fragmentation – whether GSM radiation has any effect on the actin-cytoskeleton network, the disorganization of which is another characteristic aspect of cell death in the egg chambers that was not investigated in our previous experiments [9,40]. The possible effect on the actin-cytoskeleton is examined in the present experiments, as was done previously for cell-death induction [9], during the developmental stages of early- and mid-oogenesis, *i.e.* from germarium up to stage 10, where physiological cell death does not occur.

2. Materials and methods

Our experiments were performed with *D. melanogaster*, Oregon R, wild type flies maintained under standard methods as previously described [9,32,33].

2.1. Number of F_1 pupae as a measure of reproductive capacity

According to the protocol reported previously [32,33] we placed flies that emerged within 4 h, males and females separately, in cylindrical glass vials of 2.5-cm diameter and 10-cm height, containing standard fly food. They were kept there for 2 days. The third day, when both female and male insects are sexually mature, they were transferred into a common vial with fresh food, one vial for each group, where they were kept for another 3 days, a period in which female insects provide the maximum of their oviposition [32].

The flies were exposed by the different patterns of 6-min total daily exposure to the GSM field during the first 5 days of each experiment. On the sixth day there was an additional last exposure of 6-min total duration, according to the exposure patterns described below, and 2–3 h later the parent flies were removed from the vials. The vials with the developing embryos were kept in the culture room for another 6–8 days, in order to count the number of F_1 pupae developed in each vial.

Removed maternal flies obtained from the above procedure were then anaesthetized and dissected and their egg chambers from germarium to stage 10 were prepared for acridine-orange staining and the TUNEL assay (terminal transferase-mediated dUTP nick-end labelling) in order to detect DNA fragmentation. Egg chambers of stages 11–14 were excluded as before [9], since cell death takes place physiologically anyway in the nurse and follicle cells during these stages. Rhodamine-conjugated phalloidin staining was used to test whether GSM radiation under these exposure conditions also affects the actin-cytoskeleton organization of the cells. Finally, we examined whether follicles with TUNEL-positive signal in their nurse cells also had alterations in their actin-cytoskeleton. For this purpose we used double-staining with rhodamine-conjugated phalloidin and the TUNEL assay on the same sample. Samples were examined and photographed with a Nikon Eclipse TE 2000-S fluorescence microscope and a Nikon EZ-C1 confocal laser-scanning microscope (CLSM) (Nikon Instruments, Japan).

2.2. Exposure system

A digital mobile phone handset connected to GSM-900-MHz network was used (SAR value for human head given by the manufacturer, 0.64 W/kg), in order to analyze the effects of real exposure conditions. This was the reason why we did not expose the insects under a constant power density or SAR. Real GSM signals emitted by common mobile phone handsets are never constant since there are continuous changes in their intensity and frequency [9,32,33,38–41].

The flies were exposed always at the same place for 6 min daily to mobile phone radiation modulated by human voice (speaking emission, with the same voice reading always the same text). The device was fully charged and the antenna of the mobile phone was in contact with the glass vials, parallel to the vial axis. The exposures were done following one continuous and four different types of intermittent pattern, as shown in Table 1. Each group was exposed separately, by the same mobile

Table 1
Continuous and intermittent exposure protocols.

Group	Type of exposure	Number of exposures	Duration of each exposure (min)	Duration of interval
C	Continuous	1	6	No interval
I_1	Intermittent	6	1	10 min
I_2	Intermittent	3	2	10 min
I_3	Intermittent	2	3	10 min
I_4	Intermittent	2	3	6 h
SE	Continuous	1	6	No interval

phone handset. The flies were not anaesthetized during the exposures. As reported previously [9], the 6-min total duration of daily exposure was chosen in order to have comparable conditions with the current exposure criteria [55]. In addition, our previous work has shown that a few minutes of daily exposure for only a few days is enough to produce a significant effect on insect reproduction [9,32,33].

The following types of exposure were used:

The continuous (C) type of exposure was one 6-min continuous exposure (1×6 min). The intermittent (I_1, I_2, I_3) exposure types employed 6 exposures of 1 min each (6×1 min), 3 exposures of 2 min each (3×2 min), and 2 exposures of 3 min each (2×3 min), respectively. The interval duration between the different exposure sessions was 10 min. Finally, the exposure type I_4 comprised 2 exposures of 3 min each (2×3 min) with a 6-h interval (Table 1).

In each experiment the insects were divided into six groups. The first (named C) was exposed by the C-type of exposure. The second (named I_1) was exposed by the I_1 type of exposure, etc, the fifth (named I_4) was exposed by the I_4 type of exposure and the sixth was the sham-exposed (SE) group. The SE groups were treated exactly as the exposed ones but the mobile phone was turned off during the 6-min "exposures". The daily "exposures" of the SE groups were continuous during 6 min, since preliminary experiments had already shown that there is no statistically important difference in reproductive capacity between SE groups of different sham-exposure patterns (data not shown). Each group consisted of nine male and nine female newly eclosed insects, as described before [9,32,33].

The mean power density measured for the 6-min speaking emission with the mobile phone antenna being in contact with the glass wall was 0.35 ± 0.07 mW/cm^2. The above-mentioned measurements were made with the RF Radiation Survey Meter, NARDA 8718, with its probe inside a glass vial similar to the ones we used in our earlier studies [32]. The intensities of the ELF electric and magnetic fields, due to the pulse-repetition frequency of 217 Hz, were 18.6 ± 2.7 V/m and 0.75 ± 0.17 mG, respectively, using the HOLADAY HI-3604 ELF Survey Meter. The above-measured values are the average of eight separate measurements of each kind ± standard deviation (S.D.). These values are within the current exposure criteria [55]. As explained previously [9,32,33,38–41], we have chosen to refer to the radiation in terms of its intensity, which can be objectively measured, rather than in terms of SAR, which can never be accurately estimated, especially for small insects [38].

2.3. Acridine-orange staining

Ovaries were dissected in Ringer's solution and separated into individual follicles. The follicles were incubated in 1.6 μM acridine-orange (Invitrogen, USA, A3568) in Ringer's solution for 5 min in the dark. The follicles were then washed three times for 5 min in Ringer's solution in the dark and immediately mounted on vidal glass slides in fresh Ringer's solution. The whole process from dissection to the end of the viewing did not last more than 20 min.

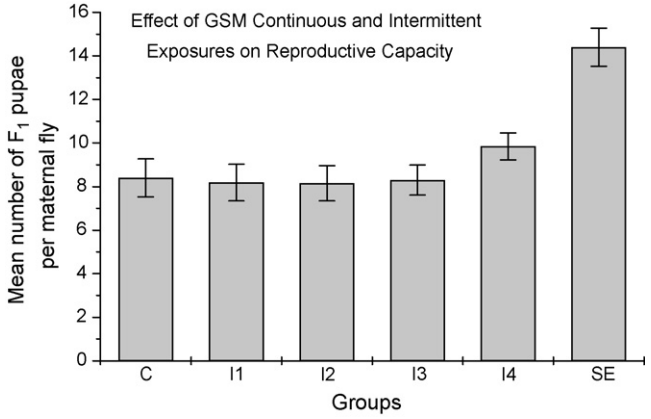

Fig. 1. Mean numbers of F_1 pupae of continuously and intermittently exposed groups and sham-exposed groups.

2.4. TUNEL assay

The TUNEL assay was performed as previously described [9].

2.5. Rhodamine-conjugated phalloidin staining

Ovaries were dissected in Ringer's solution, fixed in PBS (Invitrogen, USA, 70013-016) containing 4% formaldehyde (Polysciences, Inc., Warrington, PA, 18814) for 20 min, and permeabilized for 35 min in PBS containing 4% formaldehyde plus 0.1% Triton X-100. The follicles were then stained for 2 h in PBS containing 1 mg/ml rhodamine-conjugated phalloidin (Invitrogen, USA, R415) and washed three times (5 min each) in PBS. Finally, the samples were mounted in 90% glycerol containing 1,4-diazabicyclo (2,2,2) octane (Sigma Chemical Co., Germany) to avoid fading (anti-fading mounting medium).

2.6. Double-staining with rhodamine-conjugated phalloidin and TUNEL

Ovaries were dissected in Ringer's solution, fixed in PBS containing 4% formaldehyde for 20 min, and permeabilized for 35 min in PBS containing 4% formaldehyde plus 0.1% Triton X-100. The follicles were then stained for 2 h in PBS containing 1 mg/ml rhodamine-conjugated phalloidin (Invitrogen, USA, R415) and washed three times (5 min each) in PBS. Follicles were then incubated with PBS containing 20 μg/ml proteinase-K for 10 min. The detection of fragmented genomic DNA was performed with the *in situ* cell-death detection kit (Roche, Mannheim, Germany, 11684795910) by use of fluorescein-labeled dUTP for 3 h at 37 °C in the dark. After

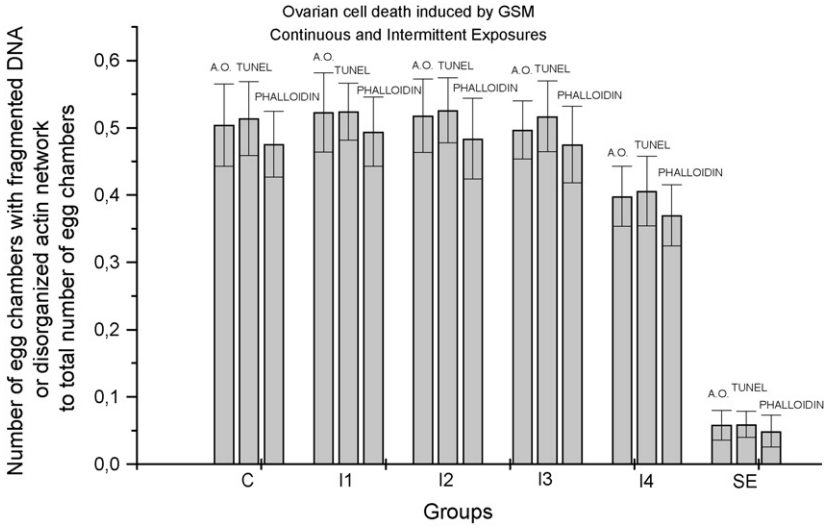

Fig. 2. Mean ratio of ovarian cell death in C, I_1, I_2, I_3, I_4 (exposed) and SE (sham-exposed) groups ± S.D., after acridine-orange (AO) Staining, TUNEL assay and phalloidin staining (number of egg chambers with fragmented DNA or disorganized actin network *vs* total number of egg chambers).

Table 2
Effect of the different GSM-900-MHz intermittent and continuous exposures on the reproductive capacity of the insect *Drosophila melanogaster*.

Experiment no.	Groups	Mean number of F1 pupae per maternal insect	% Deviation from sham-exposed group
1	C (1 × 6 min)	7.49	−42.56
	I_1 (6 × 1 min)	7.25	−44.40
	I_2 (3 × 2 min)	7.43	−43.02
	I_3 (2 × 3 min)	7.44	−42.94
	I_4 (2 × 3 min)	9.12	−30.06
	SE (control)	13.04	
2	C (1 × 6 min)	9.54	−37.85
	I_1 (6 × 1 min)	9.24	−39.80
	I_2 (3 × 2 min)	8.42	−45.15
	I_3 (2 × 3 min)	9.12	−40.59
	I_4 (2 × 3 min)	10.29	−32.96
	SE (control)	15.35	
3	C (1 × 6 min)	8.55	−39.19
	I_1 (6 × 1 min)	8.75	−37.77
	I_2 (3 × 2 min)	9.13	−35.06
	I_3 (2 × 3 min)	8.79	−37.48
	I_4 (2 × 3 min)	9.85	−29.94
	SE (control)	14.06	
4	C (1 × 6 min)	7.43	−44.59
	I_1 (6 × 1 min)	7.22	−46.16
	I_2 (3 × 2 min)	8.14	−39.30
	I_3 (2 × 3 min)	7.88	−41.24
	I_4 (2 × 3 min)	10.00	−25.43
	SE (control)	13.41	
5	C (1 × 6 min)	7.47	−49.63
	I_1 (6 × 1 min)	8.14	−45.11
	I_2 (3 × 2 min)	7.36	−50.37
	I_3 (2 × 3 min)	8.12	−45.25
	I_4 (2 × 3 min)	9.45	−36.28
	SE (control)	14.83	
6	C (1 × 6 min)	8.54	−44.26
	I_1 (6 × 1 min)	7.42	−51.57
	I_2 (3 × 2 min)	7.33	−52.15
	I_3 (2 × 3 min)	7.54	−50.78
	I_4 (2 × 3 min)	9.28	−39.43
	SE (control)	15.32	
7	C (1 × 6 min)	9.51	−36.81
	I_1 (6 × 1 min)	9.23	−38.67
	I_2 (3 × 2 min)	9.43	−37.34
	I_3 (2 × 3 min)	9.26	−38.47
	I_4 (2 × 3 min)	11.03	−26.71
	SE (control)	15.05	
8	C (1 × 6 min)	8.63	−38.45
	I_1 (6 × 1 min)	8.19	−41.58
	I_2 (3 × 2 min)	7.94	−43.37
	I_3 (2 × 3 min)	8.26	−41.08
	I_4 (2 × 3 min)	9.67	−31.03
	SE (control)	14.02	
Average ± S.D.	C (1 × 6 min)	8.40 ± 0.87	−41.63
	I_1 (6 × 1 min)	8.18 ± 0.84	−43.15
	I_2 (3 × 2 min)	8.15 ± 0.81	−43.36
	I_3 (2 × 3 min)	8.30 ± 0.69	−42.32
	I_4 (2 × 3 min)	9.84 ± 0.62	−31.62
	SE (control)	14.39 ± 0.88	

this procedure, the follicles were washed six times in PBS over the course of 90 min in the dark and mounted in anti-fading mounting medium.

The temperature during the exposures was measured as described before [9,32,33,38–41]. Statistical analysis was performed by single-factor analysis of variance test.

3. Results

The effects of continuous and intermittent exposure on the reproductive capacity of the insects, as defined by the number of F_1 pupae, and on the percentage of DNA fragmentation and actin disorganization in the egg-chamber cells are shown in Tables 2–5 and in Figs. 1–4.

3.1. Effects of continuous and intermittent exposures to GSM on the reproductive capacity

Table 2 shows the mean number of F_1 pupae of the exposed and sham-exposed insect groups from eight separate experiments. Each exposure protocol significantly decreased the number of F_1 pupae of the exposed insects compared with that of the sham-exposed groups. The C and I_1, I_2, I_3 types of exposure produced very similar decreases of the mean number of F_1 pupae (41.6%, 43.2%, 43.4%, 42.3%, respectively), whereas the I_4 procedure, with long intervals between the exposures, produced the smallest decrease in the insects' reproductive capacity (31.6%). Differences observed between C and I_1, I_2, I_3 exposed groups were within the standard

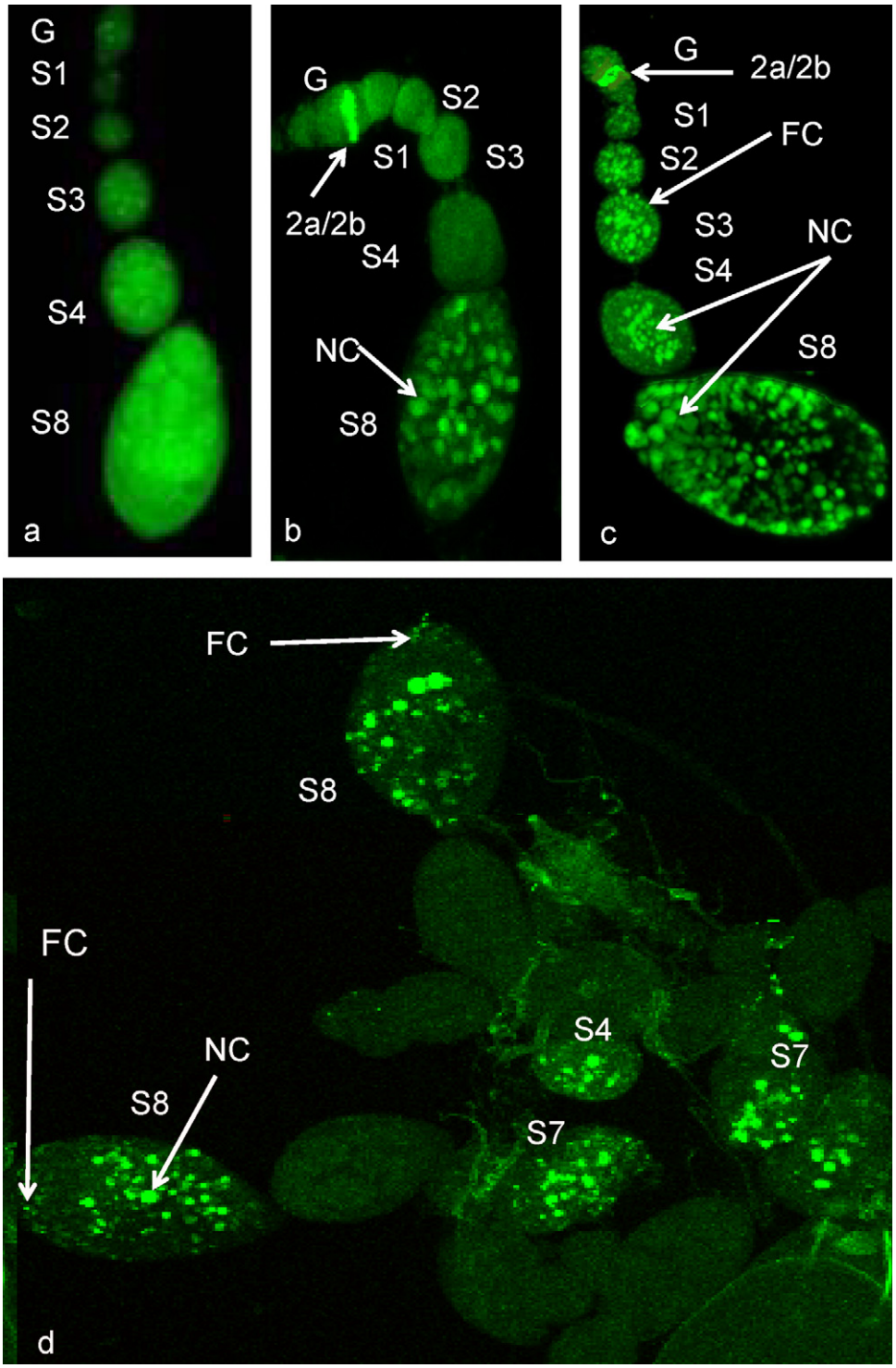

Fig. 3. Confocal micrographs of acridine-orange- and TUNEL-labeled *Drosophila melanogaster* follicles demonstrating DNA fragmentation: (a) typical ovariole of a sham-exposed insect with acridine-orange-negative signals in all the egg chambers from germarium to stage 8. (b) Ovariole of an exposed female insect (I_4 group) with acridine-orange-positive signal in the nurse cells (NC) of the two checkpoints (germarium (G) and stage 8 (S8)) and not in the intermediate stages. (c) Acridine-orange-positive signal in the nurse (NC) and follicle cells (FC) of all stages from germarium to stage-8 egg chambers from an exposed insect (I_1 group). (d) Characteristic view of egg chambers from an exposed insect (C group) with TUNEL-positive signal in the nurse and follicle-cell nuclei of stages 4–8 egg chambers and TUNEL-negative signal in other egg chambers of different stages.

deviation, but the I_1- and I_2-exposure types produced slightly larger decreases in the reproductive capacity than did I_3 and C (Table 2 and Fig. 1).

The statistical analysis showed that the probability that the C, I_1, I_2, and I_3 exposed groups differ in their reproductive capacity due to random variation is very large ($P > 0.92$). In contrast, the probability that each one of the exposed groups differs from the SE group because of random variation is in all cases negligible ($P < 10^{-8}$). Finally, the probability that each of the I_1, I_2, I_3 groups differs from the I_4 group because of random variation corresponds with a P-value of $< 10^{-3}$, whereas the corresponding probability between C and I_4 is $P < 2 \times 10^{-3}$.

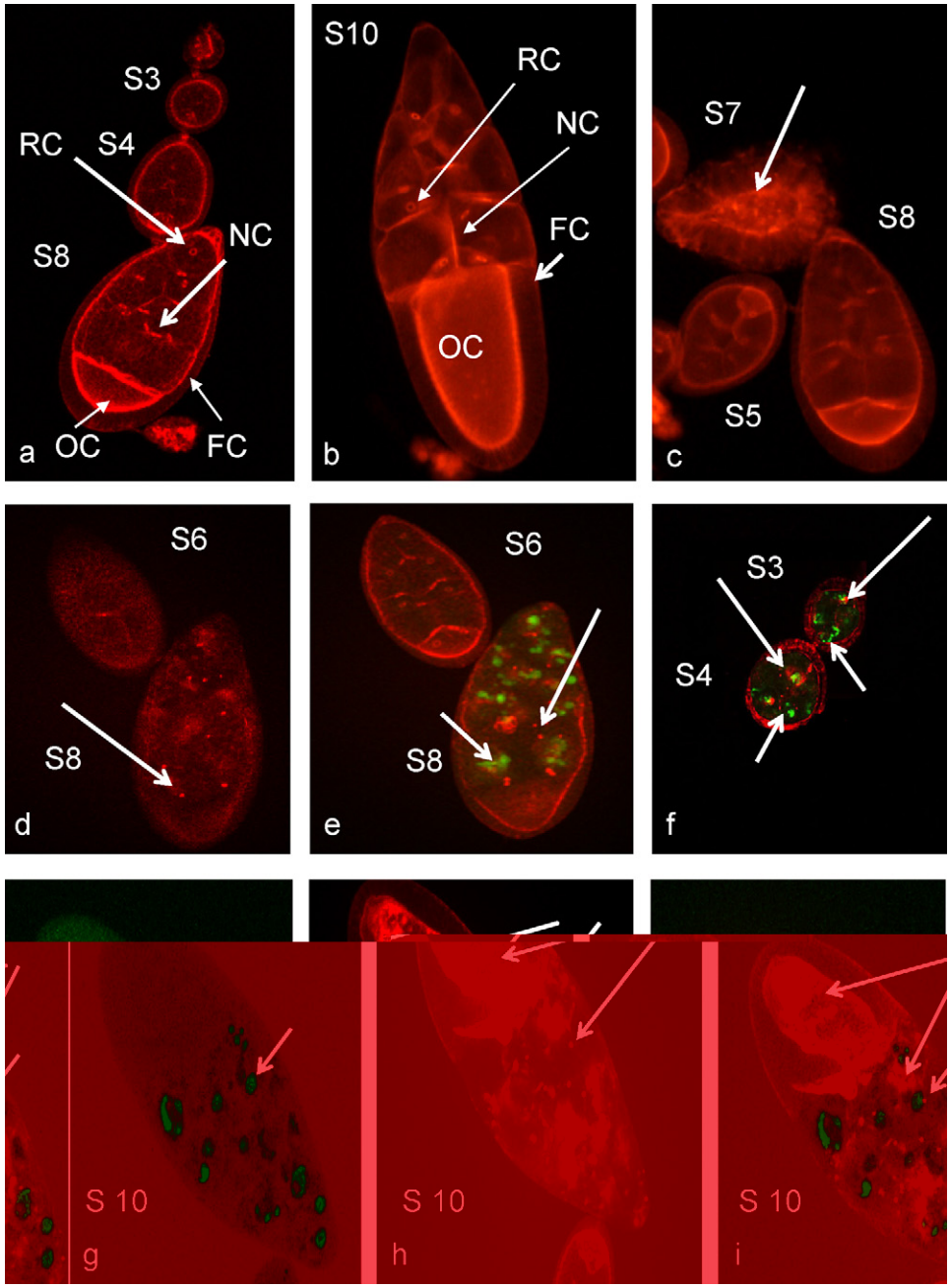

Fig. 4. Confocal micrographs of rhodamine-conjugated phalloidin-stained follicles, demonstrating the actin-cytoskeleton distribution in normal and defective egg chambers of different stages, as well as double-stained follicles with TUNEL (for DNA fragmentation) and rhodamine-conjugated phalloidin (for the actin-cytoskeleton organization). (a and b) Typical pictures of the normal actin-cytoskeleton distribution in the nurse cells (NC), oocytes (OC) and follicle cells (FC), in the egg chambers of an ovariole and a stage-10 egg chamber from a sham-exposed insect. Note the presence of the numerous ring canals (RC). (c) Characteristic picture of the actin network in a stage-7 abnormal egg chamber with disorganized actin-rich structures (arrow) and the disappearance of the ring canals obtained from an exposed female insect (I_2 group). The other two egg chambers of stages 5 and 8 show normal organization of the actin-cytoskeleton network. (d and e) Stage-8 defective egg chamber (I_2 group) with phalloidin-alone signal exhibiting disorganized actin network (d) and phalloidin-TUNEL double signal showing DNA fragmentation (short arrow) and actin disorganization (long arrow) (e). Also shown is a stage-6 normal egg chamber. (f) two egg chambers of stages 3 and 4 of an exposed insect (I_3 group) with TUNEL assay (short arrows) and phalloidin staining (long arrows) double signal for DNA fragmentation and actin disorganization, respectively. (g–i) pictures of the same stage-10 defective egg chamber (C group) with fragmented DNA (TUNEL-positive signal, short arrows) in the nurse-cell nuclei (g and i) accompanied by a completely disorganized actin network (long arrows) (h and i).

3.2. Ovarian cell death induced by continuous and intermittent exposures to GSM

The results from the acridine-orange (AO) staining, TUNEL assay, rhodamine-conjugated phalloidin staining and double-staining with rhodamine-conjugated phalloidin and TUNEL, applied to egg chambers of the C, I_1, I_2, I_3, I_4 and SE groups, are presented in Tables 3–5 and in Figs. 2–4. In each table the summarized data from four separate experiments with each assay are given. All types of exposure were found to induce cell death at all stages of the early- and mid-oogenesis (*i.e.* from germarium to stage 10), but mainly at the two checkpoints, germarium and stages 7–8.

Table 3 shows the percentages of fragmented DNA in the egg-chamber cells by AO-staining. The overall ratios of the number of egg chambers with a positive signal *vs* the total number of egg chambers at all stages (from germarium to stage 10) ± S.D. in the exposed C, I_1, I_2, I_3, and I_4 groups were 0.50 ± 0.06, 0.52 ± 0.06, 0.52 ± 0.05, 0.50 ± 0.04 and 0.40 ± 0.04, respectively.

Table 3
Influence of GSM-900-MHz intermittent and continuous exposures on DNA fragmentation visualized by acridine-orange staining.

Groups	Developmental stages	Ratio of acridine-orange-positive to total number of egg chambers of each stage	Sum ratio of acridine-orange-positive to total number of egg chambers of all stages	Percentage of acridine-orange-positive egg chambers (%)	Deviation from SE groups (%)
C	Germarium 1–6 7–8 9–10	78/92 315/714 181/286 49/144	623/1236	50.40	+44.59
I_1	Germarium 1–6 7–8 9–10	82/93 320/702 190/279 45/145	637/1219	52.26	+46.45
I_2	Germarium 1–6 7–8 9–10	80/98 333/722 171/265 40/120	624/1205	51.78	+45.97
I_3	Germarium 1–6 7–8 9–10	78/91 303/735 161/242 54/131	596/1199	49.71	+43.90
I_4	Germarium 1–6 7–8 9–10	70/90 213/692 158/295 39/129	480/1206	39.80	+33.99
SE	Germarium 1–6 7–8 9–10	11/92 19/729 36/274 5/126	71/1221	5.81	

In the SE group the corresponding ratio was 0.06 ± 0.02. The probabilities that the differences between each exposed group and the SE group are due to random variation were in all cases $P < 10^{-4}$.

TUNEL labelling (Table 4) revealed the percentage of DNA fragmentation in the ovarian cells. In the C, I_1, I_2, I_3, and I_4 exposure protocols the overall ratios of the number of egg chambers with DNA fragmentation *vs* the total number of egg chambers ± S.D., were 0.51 ± 0.05, 0.52 ± 0.04, 0.53 ± 0.05, 0.52 ± 0.05 and 0.41 ± 0.05, respectively, whereas in the SE group the corresponding percentage was 0.06 ± 0.02. The probabilities that the differences between each exposed group and the SE group are due to random variations were found again in all cases $P < 10^{-4}$.

The percentage of the egg chambers with a disorganized actin network was determined with rhodamine-conjugated phalloidin staining and is shown in Table 5. For the C, I_1, I_2, I_3, and I_4 groups the overall ratios were 0.48 ± 0.05, 0.49 ± 0.05, 0.48 ± 0.06, 0.47 ± 0.06 and 0.37 ± 0.05, respectively, while for the SE group the corre-

Table 4
Influence of GSM-900-MHz intermittent and continuous exposures on DNA fragmentation, determined by use of the TUNEL assay.

Groups	Developmental stages	Ratio of TUNEL-positive to total number of egg chambers of each stage	Sum ratio of TUNEL-positive to total number of egg chambers of all stages	Percentage of TUNEL-positive egg chambers (%)	Deviation from SE groups (%)
C	Germarium 1–6 7–8 9–10	79/90 329/718 181/302 54/141	643/1251	51.40	+45.53
I_1	Germarium 1–6 7–8 9–10	80/94 330/724 188/282 49/134	647/1234	52.43	+46.56
I_2	Germarium 1–6 7–8 9–10	85/98 322/695 183/275 47/144	637/1212	52.56	+46.69
I_3	Germarium 1–6 7–8 9–10	79/97 345/722 178/295 53/153	655/1267	51.70	+45.83
I_4	Germarium 1–6 7–8 9–10	68/91 221/696 162/288 41/138	492/1213	40.56	+34.69
SE	Germarium 1–6 7–8 9–10	12/94 16/723 37/285 8/141	73/1243	5.87	

Table 5
Influence of GSM-900-MHz intermittent and continuous exposures on actin-cytoskeleton disorganization after rhodamine-conjugated phalloidin staining.

Groups	Developmental stages	Ratio of egg chambers with disorganized actin network to total number of egg chambers of each stage	Sum ratio of egg chambers with disorganized actin network to total number of egg chambers of all stages	Percentage of egg chambers with disorganized actin network (%)	Deviation from SE groups (%)
C	1–6	321/743	565/1186	47.64	+42.71
	7–8	193/288			
	9–10	51/155			
I_1	1–6	328/739	572/1157	49.44	+44.51
	7–8	189/274			
	9–10	55/144			
I_2	1–6	312/756	569/1175	48.43	+43.50
	7–8	194/294			
	9–10	63/125			
I_3	1–6	316/759	574/1208	47.52	+42.59
	7–8	199/291			
	9–10	59/158			
I_4	1–6	233/729	436/1178	37.01	+32.08
	7–8	154/307			
	9–10	49/142			
SE	1–6	17/734	58/1177	4.93	
	7–8	32/292			
	9–10	9/151			

sponding ratio was 0.05 ± 0.02. In the case of phalloidin staining (Table 5) there are no percentages given for the germarium, since it is not possible to detect the actin network within. This is the reason why the percentages of actin disorganization (Table 5) are smaller than the corresponding percentages of DNA fragmentation (Tables 3 and 4 and Fig. 2). The probabilities that the differences between each exposed group and the SE group are due to random variation are in all cases again $P < 10^{-4}$.

A typical image of an ovariole of a sham-exposed insect (SE group) with an AO-negative signal in all the egg chambers from germarium to stage 8, is shown in Fig. 3a. Corresponding images with TUNEL-negative signal showed similar characteristics (data not shown).

Fig. 3b shows an ovariole of an exposed insect from the I_4 group with and AO-positive signal in the nurse cells of the two checkpoints, region 2a/2b of the germarium and stage 8. This was a common picture for the majority of the ovarioles of female insects from all the exposed groups. Corresponding images with TUNEL-positive signal had similar characteristics (data not shown) [9].

Fig. 3c shows an ovariole of an exposed insect (I_1 group) containing egg chambers from germarium to stage 8, with AO-positive signals in the nurse and follicle cells of all stages. Corresponding pictures obtained with the TUNEL assay had similar characteristics (data not shown).

Fig. 3d shows six egg chambers of stages 4–8 from exposed insects (C group) with TUNEL-positive signals in their nurse-cell and follicle-cell nuclei among a number of egg chambers with TUNEL-negative signals. This is a representative picture for the ratio of the number of egg chambers with induced cell death *vs* the total number of egg chambers of the female insects of the exposed groups.

Fig. 4a–c shows confocal micrographs taken after rhodamine-conjugated phalloidin staining of the follicles, which show normal and defective ovarioles and egg chambers of stages 3–10. Fig. 4a and b shows a representative ovariole and a stage-10 egg chamber from sham-exposed insects, with a normal distribution of the actin-cytoskeleton network in the nurse cells, oocytes and follicle cells, as well as the presence of the numerous ring canals (SE group).

Fig. 4c shows egg chambers of exposed insects (I_2 group). Two normal egg chambers of stages 5 and 8 are visible, and one defective egg chamber of stage 7, in which the disorganized actin-rich structures are accompanied by the disappearance of the ring canals.

Further analysis of the characteristics of the egg chambers of the exposed groups, by double-staining with TUNEL and rhodamine-conjugated phalloidin revealed that DNA fragmentation and actin-cytoskeleton disorganization coincide in the defective egg chambers, as shown in Fig. 4d–i. Confocal micrographs in Fig. 4d and e show the same two egg chambers, one normal (stage 6) and one abnormal (stage 8), with a completely disorganized actin pattern (disorganized actin-rich structures, absence of ring canals – Fig. 4d and e) – associated with fragmented nurse-cell nuclei (TUNEL-positive signal – Fig. 4e). Similar results can be seen in the confocal micrographs in Fig. 4f–i. In Fig. 4f two egg chambers are seen of stages 3 and 4 with TUNEL- and phalloidin-positive signals. In Fig. 4g–i the same is observed: a stage-10 egg chamber with TUNEL-only positive signal (Fig. 4g), phalloidin-alone signal for actin disorganization (Fig. 4h), and both signals combined (Fig. 4i).

No temperature increases were detected during the exposures, as expected according to our previous experiments [9,32,33,38–41].

4. Discussion

The results of this study correlate with our previous findings that acute exposure to GSM signals brings about DNA fragmentation in the ovarian cells and consequently a large reduction in the reproductive capacity of the insects [9,32,33,38–41]. Except for the effect of mobile phone radiation on DNA, our results show that this type of radiation also affects the actin-cytoskeleton of the egg chambers, the disorganization of which is another characteristic aspect of cell death. The induced DNA fragmentation and the induced alterations in the actin-cytoskeleton seemed to coincide in the affected follicles. The effects were again non-thermal, in agreement with our previous results [9,32,33,38–41].

In addition to our recent results [38] that the biological activity of mobile phone radiation depends on the exposure duration, the findings of the present study indicate that the influence of a certain daily dose of GSM radiation on *Drosophila*'s reproductive system depends on the exposure protocol, the intervals between the exposures, and on the corresponding time given for recovery. The exposure protocols used in this study show that C and I_1, I_2, I_3 exposures were almost equally active (Tables 2–5), since they produced similar levels of fragmented DNA and disorganized actin networks in the egg-chamber cells of the early- and mid-oogenesis, as well as a consequent decrease in the number of F_1 pupae. This result possibly suggests that the effects depend on the total amount of daily dose regardless of the intervals. I_4 exposure was found to be less bioactive than the other exposure protocols, due to its longer intervals between the exposure sessions (Tables 2–5 and Figs. 1 and 2), suggesting that recovery mechanisms are possibly initiated between exposures, similar to the situation where living organisms are exposed to fractionated, small doses of ionizing radiation [56,57].

In the case of ionizing radiation, it is well known that the longer the intervals between doses, the more successful the DNA repair [56,57]. With regard to non-ionizing radiation, Nikolova et al. [42] reported that the observed effects of EMF (changes in the transcript level) on neural progenitor cells were mitigated through activation of compensatory mechanisms.

In the present experiments, the effects were possibly mitigated in the I_4 groups by a putative compensatory mechanism that replaces the defective egg chambers by new ones. This may be the reason why the decrease in reproductive capacity (Table 2) is slightly smaller than the corresponding effect of the induced cell death (Tables 3–5). Ovaries from the C, I_1, I_2, and I_3 groups were dissected 2–3 h after the last exposure of 6-min total duration. During this period the largest numbers of defective egg chambers can be detected, whereas a few hours later the damaged egg chambers have been phagocytosed by neighbouring follicle cells and new non-defective egg chambers have been produced. In the I_4 group the ovaries were dissected 2–3 h after the last (3-min) exposure. This means that a number of defective egg chambers from the first (3-min) exposure of the same day, which took place 6 h before the last one, could not be detected. This is possibly the reason why the percentage of egg chambers showing induced cell death in this group is smaller than the corresponding values for the other exposed groups (Tables 3–5).

Apart from its effect on oogenesis, exposure to GSM also decreases the reproductive capacity of the male insects [32]. We can therefore hypothesize that the observed effects depend on the efficacy of possible compensatory mechanism in the male insects as well.

As expected, in all cases with all types of exposure, the germarium and the stages 7–8 proved to be the most sensitive stages in their response to the GSM exposure [9,40,51,54,58].

Furthermore, there is some evidence that the I_1 and I_2 intermittent exposures were possibly slightly more effective than I_3 and C, as shown by the slightly stronger effect on the reproductive capacity and on the induced cell death (Tables 2–5), although the differences were within the standard deviation. This possibly indicates that an increased number of repetitive exposures were probably more stressful for the organisms than the I_3 intermittent and the C continuous exposure patterns of the same total duration. It is also possible that in this case the increased number of repetitive stresses (more complicated exposure pattern) did not allow the organisms to initiate adaptive responses.

Based on previous similar findings with the same biological system, our laboratory has proposed a biophysical mechanism to explain the influence of EMFs on cells. According to this theory, the ELF components of GSM fields are able to alter cell function through their interaction with cell membranes. More specifically, the ELF components of the GSM fields are able to affect the regular gating of the membrane ion channels, and consequently bring about broad changes in many aspects of the cell function as well as in the process of oogenesis and reproductive capacity of the insects [59–61].

The results of the present study are in agreement with findings of several other research groups, demonstrating EMF effects related to DNA damage, alterations of the actin network, or induced apoptotic cell death in various biological systems [6,24,28,62]. Our results are also in agreement with findings of other studies that tested the comparative effects between intermittent and continuous RF or ELF exposure [6,37]. However, as already mentioned, other research groups did not report effects of these exposures [19–22].

The results of the present work demonstrate the induction of two cell-death features, DNA fragmentation and actin-cytoskeleton disorganization in the egg-chamber cells of the early- and mid-oogenesis, and subsequently a decrease in the number of F_1 pupae due to continuous or intermittent exposure to GSM-900 MHz radiation. It is worth mentioning that both features were found to coincide in the defective follicles. The disorganization of the actin network is considered to be a characteristic feature of apoptotic cell death in a large variety of cell types, as reported by others [63,64] and by our lab [51], but the possibility of necrosis cannot be excluded.

In order to confirm the interference of exposure to EMF with apoptosis, more aspects of apoptotic cell death in ovarian cells, such as caspase activation and chromatin condensation, need to be studied. Except for the induction of apoptosis, the possible link of EMF exposure with necrosis is still under investigation.

Conflict of interest

None.

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