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Detection of irradiated quail meat by using DNA comet assay and evaluation of comets by image analysis

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ABSTRACT

A simple technique of microgel electrophoresis of single cells (DNA comet assay) was used to detect DNA comets in irradiated quail meat samples. Obtained DNA comets were evaluated by both photomicrographic and image analysis. Quail meat samples were exposed to radiation doses of 0.52, 1.05, 1.45, 2.00, 2.92 and 4.00 kGy in gamma cell (gammacell ^{60}Co, dose rate 1.31 kGy/h) covering the permissible limits for enzymatic decay and stored at 2 °C. The cells isolated from muscle (chest, thorax) in cold PBS were analyzed using the DNA comet assay on 1, 2, 3, 4, 7, 8 and 11 day post irradiation. The cells were lysed between 2, 5 and 9 min in 2.5% SDS and electrophorosis was carried out at a voltage of 2 V/cm for 2 min. After propidium iodide staining, the slides were evaluated through a fluorescent microscope. In all irradiated samples, fragmented DNA stretched towards the anode and damaged cells appeared as a comet. All measurement data were analyzed using BS 200 ProP with software image analysis (BS 200 ProP, BAB Imaging System, Ankara, Turkey). The density of DNA in the tails increased with increasing radiation dose. However, in non-irradiated samples, the large molecules of DNA remained relatively intact and there was only minor or no migration of DNA; the cells were round or had very short tails only. The values of tail DNA%, tail length and tail moment were significantly different and identical between 0.9 and 4.0 kGy dose exposure, and also among storage times on day 1, 4 and 8. In conclusion, the DNA Comet Assay EN 13784 standard method may be used not only for screening method for detection of irradiated quail meat depending on storage time and condition but also for the quantification of applied dose if it is combined with image analysis. Image analysis may provide a powerful tool for the evaluation of head and tail of comet intensity related with applied doses.

1. Introduction

The radiation treatment of foods like meat, seafood and poultry is gaining importance throughout the world (Diehl, 1995; Molins, 2001; ICGFI, 2001). This process decreases or eliminates food-borne pathogens such as *Salmonella, Campylobacter, Escherichia coli* O157:H7 and *Clostridium* and protozoan parasites such as *Toxoplasma gondii* (Anellis et al., 1977, 1979; Dubey et al., 1986; FDA, 1997; FSIS 1999; Brito et al., 2002), thereby reducing food-borne illness and their associated medical and productivitiy cost (WHO, 1994, 1999). The microbiological safety of meat and meat products has received increased attention from regulators, consumers, researchers, industry and the media (Nutsch et al., 2000; Molins, 2001; WHO, 1999; Delincée, 1998). Irradiation processing can be used as a valuable method of food preservation. The process has the purpose of achieving partial or complete inactivation of cells of specific pathogens or of potential spoilage microorganisms that may be naturally present on unprocessed foods (WHO, 1999).

Analytical detection of radiation treatment of food is an important means to implement such control, once the food items have left the irradiation facility (Khan et al., 2003). Ten international standards regarding different detection procedures for irradiated food have been adopted by the European Committee for Standardization (CEN) and are now available to food control agencies (Marín-Huachaca et al., 2005).

One of these methods is the DNA Comet Assay EN 13,784 (CEN, 2001) which has been described as a rapid and inexpensive screening test to identify radiation treatment of food (Cerda et al., 1997). If the test is carried out under neutral conditions, mainly DNA double-strand breaks are observed, and on electrophoresis of single cells the DNA fragments migrate out of the cells forming a tail in the direction of the anode giving the damaged cells the appearance of a comet (Marín-Huachaca et al., 2005). With increasing radiation dose more DNA fragmentation occurs, and these fragments migrate further during the electrophoresis. Thus,

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irradiated cells will show an increased extension of the DNA from the nucleus towards the anode, whereas unirradiated cells will appear nearly circular or with only slight tails (Delincée, 2002; Marín-Huachaca et al., 2005).

The comet assay (single-cell gel electrophoresis) is widely used to evaluate DNA damage and repair in eukaryotic cells. The popularity of this test is due to its sensitivity, relatively low cost and simplicity. The most common way to visualize the comets is by using a fluorescent dye such as ethidium bromide, DAPI, acridine orange and propidium iodide. These dyes are easy to use, as they do not require special treatment of gel or time-consuming procedures, but the use of fluorescent microscope represents for less-developed laboratories a serious limitation for the use of the assay (García et al., 2007). In this rapid and simple method, after silver staining (Delincée, 1995) the migration patterns of DNA indicate a possible radiation treatment.

The classification of comet cells from type 1–5 based on morphological basis was reported by Marín-Huachaca et al. (2005). Quantification of DNA damage in individual cells based on the migration of DNA in electrical field was first described by Ostling and Johanson (1984). The measurement of DNA damage as ratio of fluorescence intensities without any assumption of the morphological shape of investigated comet was reported by Bocker et al. (1997).

There is a limited information available on the detection of irradiated quails meat by using DNA comet assay. DNA Comet Assay EN 13,784 (CEN, 2001) is a screening test to identify the irradiated food not the applied dose. The present work describes the using of image analysis to avoid individual analyser variation for the evaluation of comets corresponding to applied dose and identification of irradiated quail meat based on storage time and conditions.

2. Materials and methods

2.1. Samples

Alive quail was purchased from a pet shop in Ankara, Turkey. It was slaughtered in laboratory and quail samples, 5.0 g chest sample for each dose level, were packed in polyethylene bags, labelled and identified with its respective irradiation doses.

2.2. Irradiation

The quail meat samples were exposed at different dose levels of 0.52, 1.05, 1.45, 2.00, 2.92 and 4.00 kGy in gamma cell (gammacell ^{60}Co, dose rate 1.31 kGy/h) at Saraykoy Nuclear Research and Training Center. Harwell Amber 3042 dosimeters were used for the measurement of radiation dose. Immediately after irradiation, samples were stored in refrigerator ($4\,^{\circ}C \pm 2$) for 8 days, then they were kept at room temperature for analysis on days 9, 10 and 11.

2.3. Preparation of single-cell suspension

For each sample, about 1.0 g of very thin slices of meat were cut with a scalpel from the quail, transferred to a small beaker with 5 mL of ice-cold phosphate-buffered saline (PBS) and stirred for 5 min at about 500 min^{-1}. The suspensions were filtered first through 500 μm and then through 200 μm cloth sieves, and left to settle on ice for about 5 min. The supernatant was used as a cell suspension. Cell suspension (100 μL) was mixed with 1 mL of low-melting agarose (0.8% in PBS). A 100 μL of this mixture was spread on precoated slides.

2.4. DNA comet assay

The DNA Comet Assay for these cell suspensions was carried out as described in the European Standart EN 13,784 (CEN, 2001). The coated slides were immersed in lysis buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 2–9 min. Using the same buffer but devoid of SDS, electrophoresis was performed at 2 V/cm for 2 min. Propidium iodide staining was employed to visualize DNA. Slides were examined using a microscope (Olympus BX 51 model with flourescence and DIC attachments) at magnification 20×10 by digital color video camera (Pixera).

2.5. Image analysis

The DNA comets were evaluated by measuring the tail length, tail moment and tail DNA% of 44 comets (14, 15 and 15 comets, respectively, on days 1, 4 and 8). All measurement data were analyzed using BS 200 ProP with software image analysis (BS 200 ProP, BAB Imaging System, Ankara, Turkey). The used comet parameters for the evaluation are tail length, tail DNA% and tail moment which are described as following. The tail length is the distance of DNA migration from the body of nuclear core (in pixels) and it is used to evaluate the extent of DNA damage. Tail DNA% is the percentage of DNA in the comet tail (sum of intensities of pixels in the tail). Tail moment is tail DNA% × tail length ((DNA% in the tail) × (tail length)).

There are semi (New Manual) and fully automatic (AutoComet) comet measurements of BAB Imaging System. The comet was put in frame and comet head was identified with mouse in the semi-automatic measurement which was used for the evaluation of comets in the presented study. The quantitative comet parameters such as tail length, tail moment and tail DNA% were available immediatly after 3 s.

3. Results

3.1. Photomicrographic analysis

The non-irradiated and irradiated meat samples of quail were clearly distinguishable just by naked eye inspection at the slide under the microscope as shown in the photographs of DNA comet assay in Figs. 1–4.

The comet cells were classified on a morphological basis as described by Marín-Huachaca et al. (2005). According to this classification short tail cells with relatively little DNA degradation were classified as type 1. Other types are: type 2, long tail; type 3, long tail wider at the end; type 4, long tail separated from the head of the comet; type 5, almost no DNA is left in the head of the comet and the tail appears as a cloud, far from the head.

3.2. Image analysis

Evaluation results of comets by image analysis are shown in Table 1. Considering the root growth, clear differences between irradiated and non-irradiated samples were observed (Table 1). The changes of comet tail DNA%, tail length and tail moment with applied doses regarding to storage days are shown in Figs. 5–7, respectively. The changes of comet tail moment with storage days in non-irradiated quail meat are presented in Fig. 8.

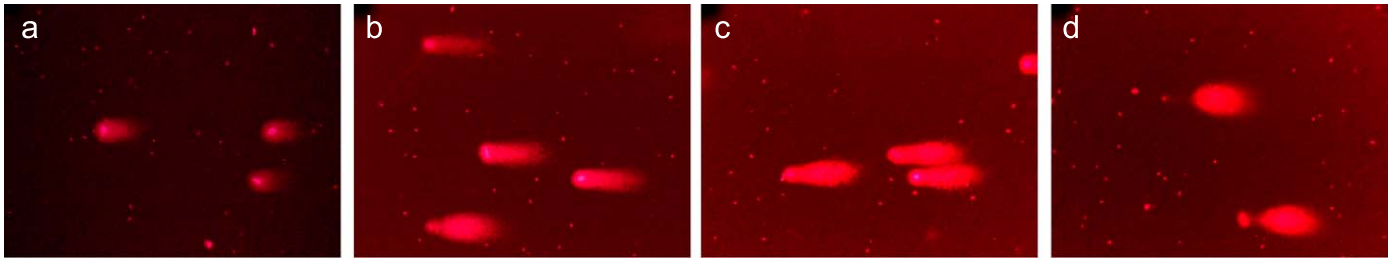

Fig. 1. Comets observed by DNA comet assay on 1-day-refrigerated quail samples. Anode to the right; propidium iodide staining; microscope objective 20 × 10. (a) Non-irradiated; (b) irradiated with 0.9 kGy; (c) irradiated with 2.0 kGy and (d) irradiated with 4.0 kGy.

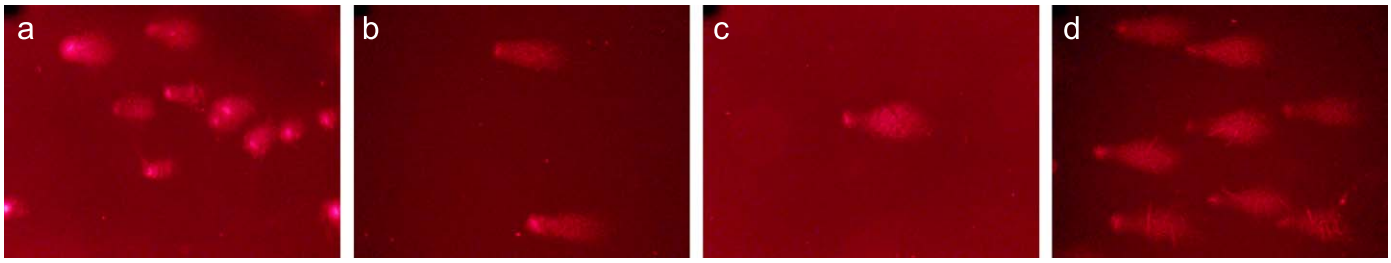

Fig. 2. Comets observed by DNA comet assay on 4-days-refrigerated quail samples. Anode to the right; propidium iodide staining; microscope objective 20 × 10. (a) Non-irradiated; (b) irradiated with 0.9 kGy; (c) irradiated with 2.0 kGy and (d) irradiated with 4.0 kGy.

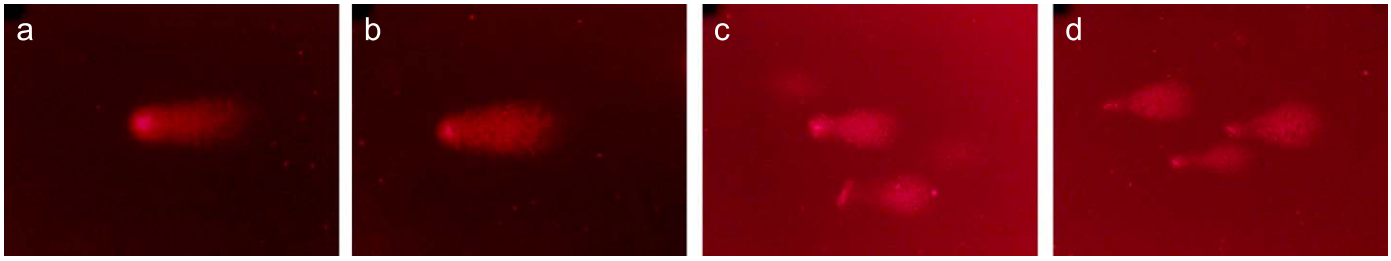

Fig. 3. Comets observed by DNA comet assay on 8-days-refrigerated quail samples. Anode to the right; propidium iodide staing; microscope objective 20 × 10. (a) Non-irradiated; (b) irradiated with 0.9 kGy; (c) irradiated with 2.0 kGy and (d) irradiated with 4.0 kGy.

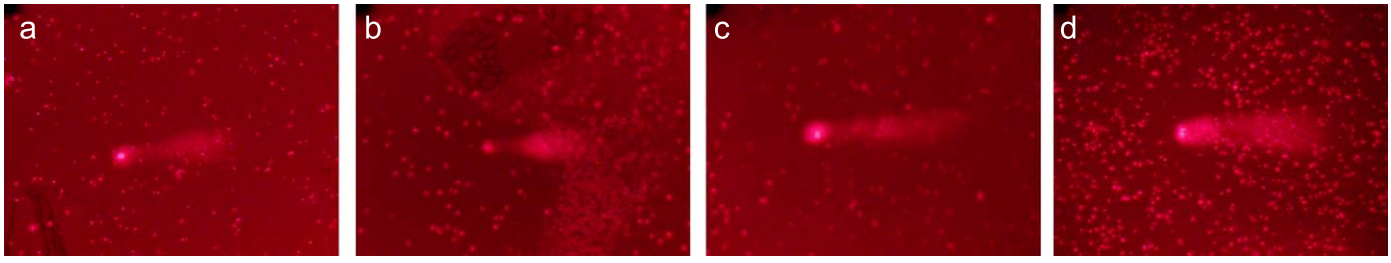

Fig. 4. Comets observed by DNA comet assay on quail samples which were stored in refrigerator for 8 days, then were kept at room temperature for analysis on day 11. Anode to the right; propidium iodide staing; microscope objective 20 × 10. (a) Non-irradiated; (b) irradiated with 0.9 kGy; (c) irradiated with 2.0 kGy and (d) irradiated with 4.0 kGy.

4. Discussion

4.1. Photomicrographic analysis

Generally in the non-irradiated samples, cells or nuclei were present as round- or oval-shaped intact images along with heads with short and faint tails. In the irradiated samples, only distinct comets were observed. All the cells were damaged and fragmented and no single intact cell could be found in any irradiated sample.

The results of microscopic observation on quail meat samples showed that the distance of DNA migration "comet length" increases with radiation dose for all samples (Figs. 1–4). Also, the amount of the irradiation dose may be indicated by the shape of the comet. Based on size and shape of the comets for different doses of radiation, a rough dose estimate may even be possible. With increasing exposure dose, the percentage of DNA in the tail rises, whereas the amount in the head decreases. In analyzing non-irradiated samples, intact cells could be observed. However, particularly day-by-day, a small quantity of different "comet" cells in non-irradiated samples was visible.

Non-irradiated samples showed intact cells with no or very short tails because there was no DNA damage or stretching of bands by electrophoresis, while irradiated samples at 0.9 kGy or

Table 1
Estimated mean values of tail DNA%, tail length and tail moment of comets by image analysis on days 1, 4 and 8 after irradiation at 0.9, 2.0 and 4.0 kGy doses.

Storage day/ irradiation dose	Tail DNA%[a]	Tail length[b]	Tail moment[c]
	Mean ± SE	Mean ± SE	Mean ± SE
Day 1			
Control	56,434 ± 0,832	48,566 ± 1,262	27,556 ± 1,058
0.9 kGy	69,746 ± 0,186	74,053 ± 1,890	51,633 ± 1,220
2.0 kGy	74,076 ± 1,956	69,130 ± 2,420	51,483 ± 3,020
4.0 kGy	87,766 ± 1,676	121,323 ± 3,380	106,820 ± 4,950
Day 4			
Control	71,603 ± 1,463	56,723 ± 1,880	40,573 ± 1,420
0.9 kGy	72,580 ± 4,150	70,706 ± 1,120	51,586 ± 3,760
2.0 kGy	74,585 ± 0,997	61,450 ± 1,310	45,835 ± 1,160
4.0 kGy	85,338 ± 0,356	106,942 ± 1,248	91,330 ± 1,350
Day 8			
Control	83,817 ± 0,757	107,532 ± 2,345	90,277 ± 2,620
0.9 kGy	82,383 ± 1,726	112,653 ± 1,913	92,996 ± 3,460
2.0 kGy	74,688 ± 1,098	126,400 ± 2,730	94,556 ± 2,010
4.0 kGy	84,333 ± 0,293	199,666 ± 0,693	168,403 ± 1,170

[a] Tail DNA% = percent of DNA in the comet tail.
[b] Tail length = length of the comet tail measured from right border of head area to end of tail (μm = pixels).
[c] Tail moment = tail DNA% × tail length ((DNA% in the tail) × (tail length)).

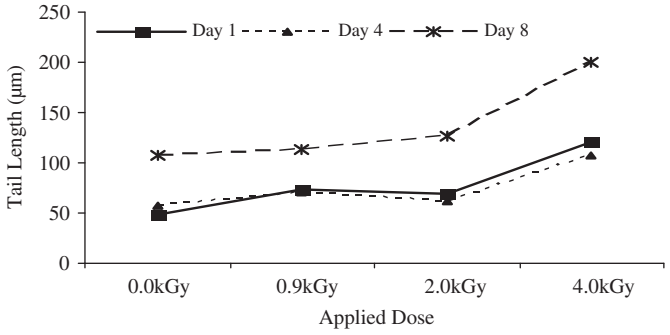

Fig. 5. Changes of comet tail DNA% with applied doses (0, 0.9, 2.0 and 4.0 kGy) regarding to storage days in quail meat.

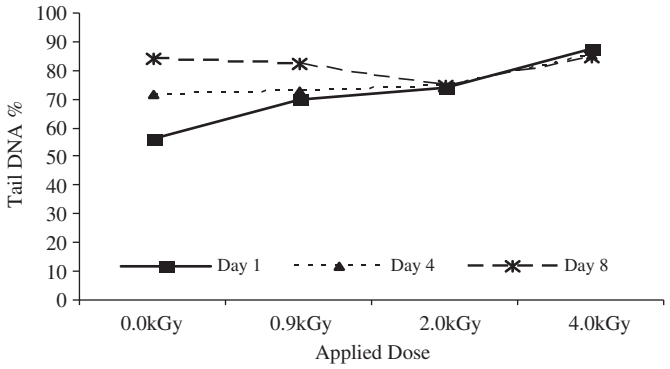

Fig. 6. Changes of comet tail length with applied doses regarding to storage days in quail meat.

more showed comet cells with long tails, representing DNA fragments migration, and a damaged appearance. Moreover, the tail length increased depending on the radiation dose. The tail end was wider and thicker than the head of the comet.

Irradiated samples clearly showed a more pronounced DNA migration than the control, permitting the control (non-irradiated

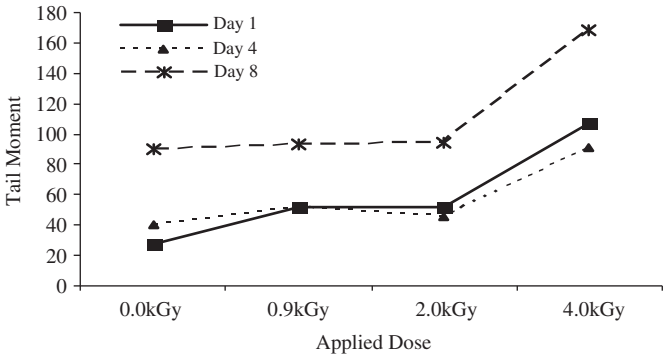

Fig. 7. Changes of comet tail moment with applied doses regarding to storage days in quail meat.

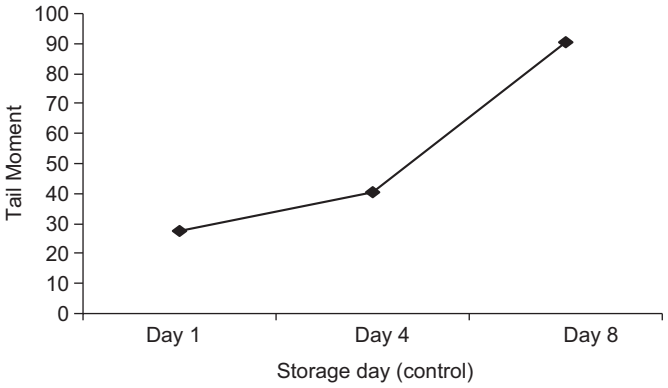

Fig. 8. Changes of comet tail moment with storage days in non-irradiated quail meat.

samples) to be distinguished from irradiated samples until 4, 8 and 11 days after treatment (Figs. 1–4).

From day 4, DNA fragmentation increased significantly as compared to the 1st day. The findings of the present study show that the DNA comet assay also can be used as a rapid method to verify the hygienic status of fresh quail. An increase in DNA migration with storage time was also observed in refrigerated and frozen beef samples (Park et al., 2002; Marín-Huachaca et al., 2005) and fresh chicken, pork and fish samples (Cerda, 1998a,b) applying the comet assay. Furthermore, studies in refrigerated exotic meats such as boar, jacare and capybara (Villavicencio et al., 2000), and in refrigerated pork (Araújo et al., 2004) and poultry (Villavicencio et al., 2004) have offered good results. Khan et al. (2003) applied the comet assay on some fresh and frozen samples of meats (lamb, beef, turkey) and fresh seafood to detect an irradiation treatment.

The shelf-life of fresh quail samples is a few days if stored in a refrigerator. Its edibility will depend on storage conditions; aerobic or vacuum packaging (anaerobic), temperature and initial amount and bacteria types in the product (Cerda, 1998a,b). Even at low temperature (2 °C) some bacteria will reproduce and eventually spoil the food (Lambert et al., 1991). Several pathogenic bacteria (e.g. *Lysteria monocytogenes*) are able to reproduce in chilled conditions and causes a health hazard (Brackett, 1992). Ionizing radiation can be used to reduce the initial load of bacteria, thus prolonging the shelf-life of the product (Lambert et al., 1991; Jay, 1992).

The non-irradiated samples were analyzed at the same intervals as the irradiated ones, that is after 1, 4 and 8 days. On day 1 the majority of the cells showed comets with very short tails (type 1, as indicated by Marín-Huachaca et al.), typical of

non-irradiated controls (Figs. 1–3), while some cells showed various degrees of DNA degradation, reflected by the shapes of the comets (e.g. long tails). Few or no bacteria were observed at this stage. In Fig. 2a, after 4 days a large number of cells showed comets with long tails (type 2). The degree of degradation increased (more fragmentation of DNA), but there were still comets with short tails (type 1), as on day 1. Bacteria were seen in small numbers in all the samples. In Figs. 1–3, after 8 days of storage in the refrigerator, the cells showed advanced stages of DNA degradation, reflected by comets with long wide tails (type 3).

Shown comets in Fig. 4 belong to quail samples which were stored in refrigerator for 8 days, then were kept at room temperature till analysis on day 11. The comet types on day 11 analysis are of type 4 or/and type 5 and the bacteria had greatly increased in number in all the samples (Figs. 1–3) and off-odour had developed.

Irradiation produces a homogeneous pattern of comets (Cerda, 1993; Cerda, 1997; Cerda et al., 1997; Koppen and Cerda, 1997). Variations would reflect the status of DNA prior to irradiation or post-irradiation storage conditions. One day after irradiation the cells showed the typical pattern of irradiated cells (comets that are 2–3 times longer than those of non-irradiated control cells). The shape of the comets corresponded to the applied doses (Figs. 1–3). Four days after irradiation there was a general increase of DNA damage caused by storage at 2 °C. Cells from all the irradiated samples (0.9, 2.0 and 4.0 kGy), thus, showed comets with larger tails than on day 1 (corresponding to higher doses than actually given). There was no increase of the bacterial population. Eight days after irradiation the cells from all the irradiated samples (0.9, 2.0 and 4.0 kGy) showed a high degree of DNA fragmentation, with comets that would correspond to very high doses (10 kGy or more) and increase of the bacterial population (Figs. 1–4).

4.2. Image analysis

Root elongation of comets was a good parameter to discern non-irradiated samples from irradiated ones when the comets evaluated by the image analysis. A larger quantity of DNA is fragmented with increasing radiation doses. The values of tail DNA%, tail length and tail moment were significantly different and identical between 0.9 and 4.0 kGy dose exposure, and also among storage times on day 1, 4 and 8 (Table 1). The values of tail DNA%, tail length and tail moment of quail samples significantly increased after 2.0 up to 4.0 kGy at all storage time (Figs. 5–7). As seen in Figs. 5–7, increases on tail DNA%, tail length and tail moment with the effect of irradiation were sharply started at 2.0 kGy. However, changes on the estimated comet parameters with applied doses for day 1, 4 and 8 showed similar pattern. Changes of tail moments with storage days showed that spoilage of quail meat samples started after 4 days storage in fridge at 2 °C (Fig. 8).

In DNA comet assay as a screening method of irradiated foods, evaluation of comets have been based on photomicrographic analysis that may be varied by analyser. Image analysis system for comet evaluation may strengthen the method by avoiding individual analyser variation.

5. Conclusions

In conclusion, quantification of comets is possible by image analysis which provides a powerful tool for the evaluation of head and tail of comet intensity related with applied doses. It was apparent during the analysis of non-irradiated fresh controls that the DNA comet assay method could also be used to control the freshness of quail meat.

The DNA Comet Assay EN 13,784 (CEN, 2001) standard screening method to identify irradiated foods may also be used as quantitative detection of applied dose if it is combined with image analysis evaluation.

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