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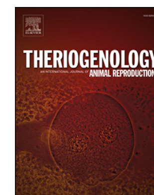

DNA damaging effect of paclitaxel in the epididymal sperms as a chemotherapeutic agent and possible remedies to prevent this effect: A study on reproductive potential of male cancer patients of reproductive age

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ABSTRACT

Cancer is a major public health problem, young cancer patients therefore undergo chemotherapy, and most of them may lose their fertility. DNA damage level provides important clues about the quality and reproductive potential of spermatozoa. In this study, we evaluated the levels of both DNA fragmentation and abnormal DNA integrity in the epididymal sperms of New Zealand rabbit (*Oryctolagus cuniculus*) after cryopreservation using the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay and the toluidine blue (TB) staining methods and assessed the effects of paclitaxel, resveratrol, L-glutamine (LG), and basal medium eagle (BME) solution on DNA damage. Paclitaxel induced the levels of both DNA damages in the sperms, but resveratrol ameliorated this effect. LG and BME supplementation to the extender prevented the sperm samples from DNA fragmentation after cryopreservation. Chemotherapy drugs containing paclitaxel can cause the sperm DNA to be damaged, and hence adversely affect the fertility of male cancer patients of reproductive age. The administration of resveratrol together with paclitaxel may ameliorate the DNA damage inducing effect of paclitaxel. Sperm banking and cryopreservation with the appropriate cryoprotectants such as LG and BME prior to cancer treatment can also be suggested to all male cancer patients of reproductive age facing cancer treatment for fertility preservation.

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1. Introduction

In general, sperms are maturated in the epididymis, stored in cauda region of epididymis until they ejaculated, and they are then able to fertilise oocytes. In addition, epididymal spermatozoa are physiologically protected by various antioxidant enzymes such as catalase and superoxide dismutase in their environment [1,2].

Cancer is a major public health problem in many parts of the world [3]. For instance, the incidence of testicular cancer in males from 15 to 45 years old nowadays reaches up to 70% [4]. Cancer itself can adversely affect the male fertility by impairing spermatogenesis, and all cancer therapies (chemotherapy, radiation, and surgery) are also potential threats to reproductive potential of males [5]. A lot of young cancer patients undergo chemotherapy, and most of them may lose their fertility; therefore, future fertility is also an area of remarkable concern for young cancer survivors [6]. Sperm recovery from the caudal epididymis of young cancer patients with microsurgical epididymal sperm aspiration as well as ejaculates and the cryopreservation of these sperms prior to initiating cancer treatment may enable these patients to father biologically related children [5].

Paclitaxel is a member of the taxane class of anticancer drugs and one of the most common chemotherapeutic agents used

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against many forms of cancer [7]. It causes reactive oxygen species (ROS) production and DNA damages, and DNA damages induced by paclitaxel are used to kill the cancer cells [8,9]. Resveratrol (3,4′,5-trihydroxystilbene), which is a phytoalexin, is found in many plants including grapes, peanuts, and berries [10] and it shows antioxidant and radical scavenging activities [11,12].

Cryopreservation is a long-term storage technique with very low temperatures to preserve the sperm of various animal species for extended period of time at a low cost. Therefore, cryopreservation of semen has been of great importance in agriculture, conservation of wild animals and endangered species, and treatment of human infertility [13]. However, it is well known that cryopreservation induces sublethal damage to the spermatozoa, which may result in loss of motility, viability, *in vivo* fertilising capacity, deterioration of acrosomal and plasma membrane integrity, and DNA damage [14–16].

The freezing-thawing process in cryopreservation results in high concentrations of ROS [17–19]. ROS can cause damage in sperm plasma membranes and in DNA [20], and it is related with defective spermatozoa and male infertility [21,22]. In some cases, sperms carrying significant levels of oxidatively damaged DNA do not lose their fertilisation capacity and can fertilise oocyte both *in vivo* and *in vitro* [23]. As a consequence of this, poor implantation and pregnancy rates, unhealthy offspring, and *de novo* mutations in germ line and post fertilisation may occur [24]. Both enzymatic and nonenzymatic antioxidants are highly found in seminal plasma [25,26], but spermatozoa do not contain protective cytoplasmic antioxidant enzymes due to their limited cytoplasm [26]. Various cryoprotectants supplemented with different antioxidants are used in sperm freezing protocols in order to protect sperm from the detrimental effects of the seminal ROS caused by the imbalance between the scavenging activity of antioxidant systems and the ROS generation [27–32].

Amino acids look like to be crucial components for sperm freezing extender due to their antioxidant properties. The most abundant amino acid in the body is L-glutamine (LG) which is the precursor of glutathione known as the most important antioxidant [33]. LG plays an important role in regulating the protein synthesis/degradation and the acid–base balance and in improving the adaptation to stress [34,35]. Basal medium eagle (BME) essential amino acid solution is classical cell culture solution supplemented with the minimal components including 13 amino acids and eight vitamins [36]. Both additives have been documented to protect the sperm against the adverse effects of cryopreservation and to improve the spermatozoa quality in various animal species [37–42], since both of them have been reported to have antioxidant activities [37,41,43].

In this study, we evaluated the levels of DNA damage in the epididymal sperms of New Zealand rabbit (*Oryctolagus cuniculus*) after cryopreservation. The objectives of this study were (i) to evaluate the effects of intravenous (IV) injections of paclitaxel and resveratrol before collection of epididymal sperms on the levels of DNA damage, (ii) to test whether addition of BME and LG to extender has a reducing effect on DNA damage after cryopreservation, and (iii) to compare the results of the terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay and the toluidine blue (TB) staining methods used for examination of DNA damage in sperm samples.

2. Materials and methods

2.1. Animals

In the present study, 32 New Zealand rabbits were used. The rabbits were 36 months old and sexually matured, and the weight of them ranged from 2.5 to 3 kg. The research procedure was approved by the Ethics Committee (SÜVFEK, 2012/031).

2.2. Experimental design

The experimental design consisted of four treatment groups (Fig. 1). Each treatment group consisted of eight rabbits. Of these treatment groups, control group (C) was administered 40 ml saline, paclitaxel group (P) was administered 5 mg/kg paclitaxel in 40 ml saline, resveratrol group (R) was administered 4 mg/kg resveratrol in 40 ml saline, and paclitaxel + resveratrol group (PR) was administered 5 mg/kg paclitaxel + 4 mg/kg resveratrol in 40 ml saline in every injection. All administrations were IV and continued once a week for 8 weeks. Since the duration of spermatogenesis is about 7–8 weeks [44], a minimum administration period of 8 weeks was considered to be enough to determine the effect of certain exogenous factors in the epididymal sperms.

2.3. Sperm collection and processing

Epididymal sperm samples were obtained from cauda epididymis of the rabbits. A Tris-based extender [250 mM Tris, 88 mM citric acid, 47 mM glucose, 50 mM sucrose, 20% egg yolk (v/v), pH 6.8] was used as the base extender (cryopreservation diluent). For each treatment group, sperm samples were pooled to increase the volume for replication and to eliminate variability among the evaluated samples. Pooled sperm samples per treatment group were divided into four portions and included in the study as replication (Fig. 1). Each replication was split into three equal aliquots and diluted at 37 °C with the base extenders containing 2.5 mM L-glutamine (LG), 5% basal medium eagle (BME), and no additive (C). Diluted sperm samples were loaded into 0.25-ml straws, sealed with polyvinyl alcohol powder, and equilibrated at 5 °C for 2 h. After equilibration, the straws were frozen in liquid nitrogen vapour, 4 cm above the liquid nitrogen, for 15 min and plunged into liquid nitrogen for storage. After stored in the liquid nitrogen for at least one week, the frozen straws were thawed individually at 37 °C for 25 s in a water bath for evaluation of the sperm samples.

2.4. Assessment of DNA damage

DNA damage was investigated separately for each sample as two different concepts: (i) DNA fragmentation caused by nicks and double-strand breaks and (ii) abnormal DNA integrity/chromatin structure. DNA fragmentation level was examined using the TUNEL assay, whereas abnormal DNA integrity level was examined using the TB staining.

2.4.1. TUNEL assay

The TUNEL assay is a method which is used for the direct detection of DNA fragmentation caused by nicks and double-strand breaks [45]. For this method, a commercial kit procedure (*In Situ* Cell Death Detection Kit, POD; ROCHE; cat: 11684817910) was followed. Briefly, sperm cells were smeared on poly-L-lysine coated slides. After that, slides were air-dried and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7, for 1 h at room temperature. The fixed sperms were washed with PBS, and permeabilised with 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 2 min on ice. Afterwards, sperms were washed with PBS and samples were incubated in a humidified atmosphere in 50 μl of TUNEL reaction mixture for 60 min at 37 °C in the dark. After washed with PBS, slides were incubated in a humidified atmosphere in 50 μl of TUNEL-POD for 30 min at 37 °C and washed with PBS. The washed slides were incubated with 100 μl of DAB

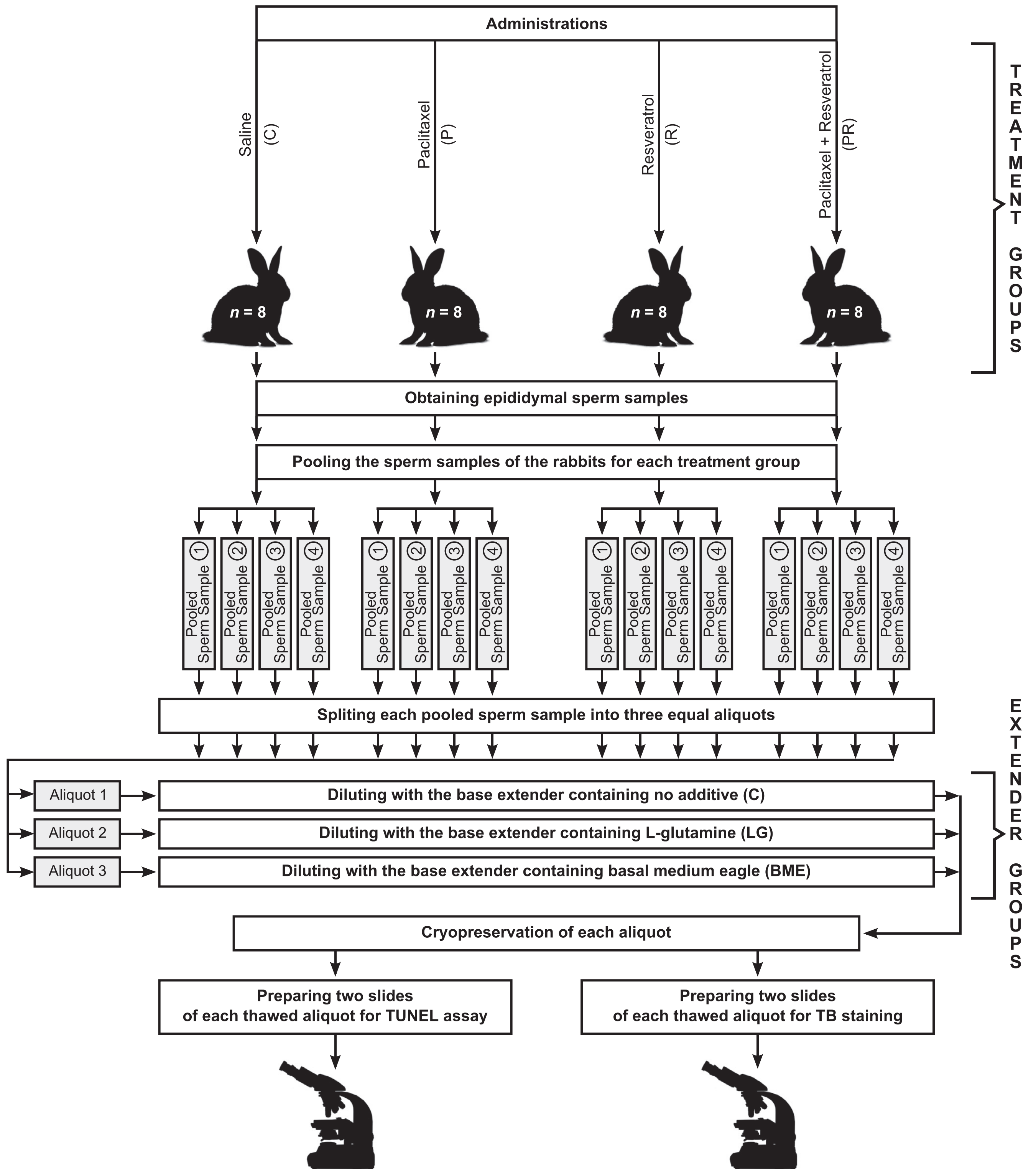

Fig. 1. Schematic demonstration of experimental design together with sperm collection and processing of the study.

substrate solution at room temperature until colour reaction. At the end of the procedure, washed slides were counter stained with methyl green. The slides were incubated with TUNEL-label solution (without terminal transferase) instead of TUNEL reaction mixture for negative controls. The percentage of TUNEL-positive cells with brown colour in their nuclear region, which is an indicator of fragmented DNA, was calculated by counting randomly selected 100 sperms from 10 different regions in each slide per sample under light microscope (Fig. 2a).

2.4.2. TB staining

The TB, a basic nuclear dye, staining is a sensitive test for incomplete DNA structure and packaging (abnormal DNA integrity) [46]. Briefly, sperm cells were smeared on poly-L-lysine coated

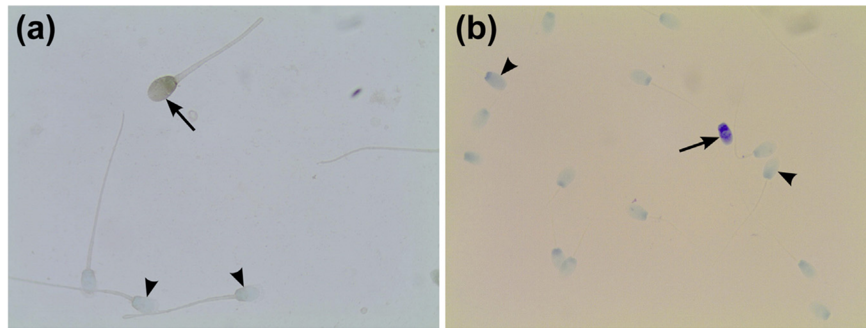

Fig. 2. Photomicrographs of epididymal sperms of New Zealand rabbits for the methods of the TUNEL assay (a) and the TB staining (b). Arrow shows the sperms with positive reaction and arrowhead shows the sperms with negative reaction for both methods (×1000).

slides, air-dried and fixed with 96% ethanol-acetone (1:1) at 4 °C for 30 min. Afterwards, slides were hydrolysed in 0.1 N HCl at 4 °C for 5 min and rinsed with distilled water. Washed slides were then stained with 0.05% TB for 10 min. The light blue sperm heads were considered as normal (which had good chromatin integrity), and violet (purple) or dark blue sperms were categorised as abnormal (diminished chromatin integrity) sperms. The percentage of TB-positive cells (violet/purple or dark blue sperms) was calculated by counting randomly selected 100 sperms from 10 different regions in each slide per sample under light microscope (Fig. 2b).

2.5. *Statistical analysis*

Results were expressed as mean ± standard deviation (SD). The differences in the numbers of the sperm cell with damaged DNA among the groups were analysed using the Kruskal-Wallis test. If Kruskal Wallis test indicated that there were significant differences among the groups, then Mann-Whitney *U* test was performed to see which group was different from the others. The correlation between the TUNEL assay and the TB staining methods was analysed using Pearson correlation test. All these statistical analyses were performed using Minitab 16 (Minitab Inc., State College, PA). All P values < 0.05 were considered to be statistically significant.

3. Results

3.1. *Treatment group evaluation*

3.1.1. *TUNEL assay*

The percentages of the C, P, R, and PR treatment groups were 6.08 ± 2.29, 11.29 ± 1.96, 4.46 ± 1.20, and 7.33 ± 2.04, respectively (Table 1 and Fig. 3). After statistical analysis of data of the groups obtained using the TUNEL assay, it was found that there were significant differences among them ($H = 30.68$; $df = 3$; $P < 0.001$). DNA damage in the P group was higher compared with those in the C, R, and PR groups ($P < 0.001$). In addition, DNA damage in the R group was lower compared with those in the C ($P < 0.05$) and PR ($P < 0.001$) groups. However, no significant difference was detected between the C and PR groups in terms of DNA damage ($P > 0.05$). It was clear that the treatment of resveratrol reduced DNA fragmentation which was caused by paclitaxel and occurred spontaneously, while the treatment of paclitaxel resulted in higher level of DNA fragmentation than the other treatments after cryopreservation.

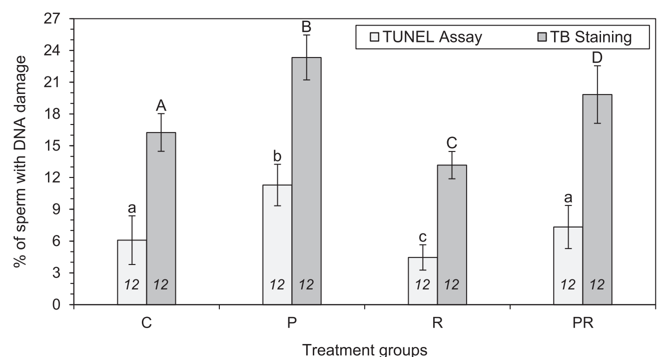

Fig. 3. DNA damages detected in the treatment groups using the TUNEL assay and the TB staining. Numbers in the columns show *n* values (the numbers of aliquots). Means that do not share a letter in each method are significantly different from each other. C: Control group, P: Paclitaxel group, R: Resveratrol group, PR: Paclitaxel + Resveratrol group.

The values of the treatment groups per extender group were also analysed statistically (Table 2 and Fig. 4a). It was found that there were significant differences among the treatment groups within the C ($H = 13.31$; $df = 3$; $P < 0.01$), LG ($H = 11.93$; $df = 3$; $P < 0.01$), and BME ($H = 10.40$; $df = 3$; $P < 0.05$) extender groups. For each extender group, the highest DNA damage was observed in the P treatment group ($P < 0.05$). For each extender group, the PR treatment group had lower DNA damage compared with the P treatment group ($P < 0.05$), but the C and R treatment groups had almost same values of DNA damage ($P > 0.05$). The PR treatment group had significantly higher DNA damage than the C and R

Table 1
Descriptive statistic data of the treatment groups for the TUNEL assay and the TB staining. *n* values indicate the number of aliquots in each treatment group.

Treatment group	TUNEL assay			TB staining		
	n	Mean (%)	Range (%)	*n*	Mean (%)	Range (%)
C	12	6.08 ± 2.29[a]	1.50–8.50	12	16.25 ± 1.78[a]	12.50–19.00
P	12	11.29 ± 1.96[b]	9.00–15.00	12	23.33 ± 2.12[b]	19.00–26.00
R	12	4.46 ± 1.20[c]	2.50–6.50	12	13.17 ± 1.29[c]	10.50–15.00
PR	12	7.33 ± 2.04[a]	4.50–11.00	12	19.83 ± 2.72[d]	15.00–25.00

Means that do not share a superscript letter within same column are significantly different from each other.

Table 2
Descriptive statistic data for the treatment groups based on extender groups for the TUNEL assay and the TB staining. *n* values indicate the number of aliquots in each treatment group.

Treatment group	Extender group	TUNEL assay			TB staining		
		n	Mean (%)	Range (%)	*n*	Mean (%)	Range (%)
C	C	4	7.63 ± 1.18^{a}	6.00–8.50	4	16.88 ± 1.25^{a}	15.50–18.50
P		4	13.13 ± 1.75^{b}	11.00–15.00	4	24.88 ± 1.11^{b}	23.50–26.00
R		4	5.25 ± 1.32^{ac}	3.50–6.50	4	13.00 ± 1.08^{c}	11.50–14.00
PR		4	9.88 ± 0.75^{d}	9.50–11.00	4	20.38 ± 4.50^{ab}	15.00–25.00
C	LG	4	6.13 ± 1.49^{a}	4.50–8.00	4	14.75 ± 1.55^{a}	12.50–16.00
P		4	10.38 ± 1.80^{b}	9.00–13.00	4	23.38 ± 1.31^{b}	21.50–24.50
R		4	3.88 ± 0.75^{a}	3.00–4.50	4	13.38 ± 1.25^{a}	12.00–15.00
PR		4	5.88 ± 1.11^{a}	4.50–7.00	4	20.50 ± 1.68^{b}	18.50–22.50
C	BME	4	4.50 ± 3.03^{a}	1.50–8.00	4	17.13 ± 1.75^{a}	15.00–19.00
P		4	10.38 ± 1.03^{b}	9.00–11.50	4	21.75 ± 2.66^{b}	19.00–24.50
R		4	$4.25 \pm 1.26^{a,*}$	2.50–5.50	4	13.13 ± 1.80^{c}	10.50–14.50
PR		4	$6.25 \pm 0.65^{a,*}$	5.50–7.00	4	18.63 ± 1.11^{ab}	17.50–20.00

Means that do not share a superscript lower-case letter within same extender group are significantly different from each other.
* Difference between two groups is statistically significant regardless of the superscript lower-case letter.

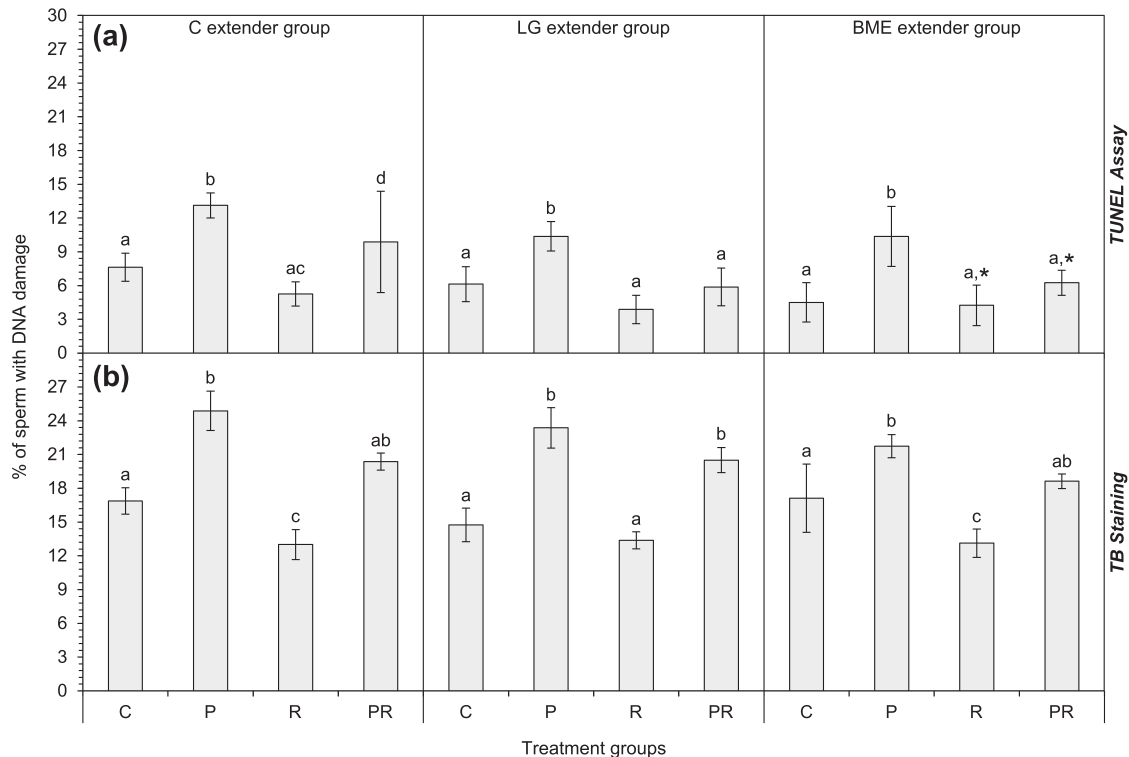

Fig. 4. DNA damages detected in the treatment groups based on extender groups using the TUNEL assay (a) and the TB staining (b). *n* value (the number of aliquots) for each treatment group was four. Means that do not share a lower-case letter in each extender group are significantly different from each other. Asterisks show that there is statistically significant difference between two groups regardless of the letter that they share. Extender groups; C: Control group, LG: L-Glutamine group, BME: Basal medium eagle group. Treatment groups; C: Control group, P: Paclitaxel group, R: Resveratrol group, PR: Paclitaxel + Resveratrol group.

treatment groups in the C extender group ($P < 0.05$). Similarly, the PR treatment group had significantly higher DNA damage than the R treatment group in the BME extender group ($P < 0.05$). It was clear that the treatment of resveratrol together with paclitaxel resulted in lower DNA fragmentation compared with the treatment of solely paclitaxel for all extender groups. Although not statistically significant for all extender groups, the treatment of resveratrol was likely to decrease the level of DNA fragmentation. This examination based on the extender groups enabled us to test the results of treatment group evaluation, and it revealed that the results were almost same with them.

3.1.2. TB staining

The percentages of the C, P, R, and PR treatment groups were 16.25 ± 1.78, 23.33 ± 2.12, 13.17 ± 1.29, and 19.83 ± 2.72, respectively (Table 1 and Fig. 3). After statistical analysis of data of the groups obtained using the TB staining, it was found that there were significant differences among them ($H = 38.03$; $df = 3$; $P < 0.001$). DNA damage in the P group was the highest among the groups ($P < 0.001$), whereas that in the R group was the lowest ($P < 0.001$). In addition, DNA damage in the C group was lower than that in the PR group ($P < 0.05$). It was clear that the treatment of resveratrol reduced the level of abnormal DNA integrity which was caused by

paclitaxel and occurred spontaneously, while the treatment of paclitaxel resulted in high level of abnormal DNA integrity after cryopreservation.

The values of the treatment groups per extender group were also analysed statistically (Table 2 and Fig. 4b). It was found that there were significant differences among the treatment groups within the C ($H = 12.18$; $df = 3$; $P < 0.01$), LG ($H = 12.84$; $df = 3$; $P < 0.01$), and BME ($H = 12.09$; $df = 3$; $P < 0.01$) extender groups. For each extender group, higher values of DNA damage were observed in the P and PR treatment group ($P < 0.05$), but there were no significant differences between the PR and C treatment groups in the C and BME extender groups ($P > 0.05$) unlike the LG extender group. The R treatment group had significantly lower DNA damage than the C treatment group in the C and BME extender groups ($P < 0.05$), but not in the LG extender group ($P > 0.05$). It was clear that the treatment of resveratrol reduced the level of abnormal DNA integrity which occurred spontaneously. Although not statistically significant for all extender groups, the treatment of resveratrol was likely to decrease the level of abnormal DNA integrity. This examination based on the extender groups enabled us to test the results of treatment group evaluation, and it revealed that the results were almost same with them.

3.2. Extender group evaluation

3.2.1. TUNEL assay

The percentages of the C, LG, and BME extender groups were 8.97 ± 3.22, 6.56 ± 2.73, and 6.34 ± 2.98, respectively (Table 3 and Fig. 5). Extender groups were examined statistically, and it was found that there were significant differences among them ($H = 6.30$; $df = 2$; $P < 0.05$). According to the statistical results, supplementation of either LG or BME to the extender before cryopreservation reduced DNA damage (6.56 ± 2.73 and 6.34 ± 2.98, respectively) compared with the C group (8.97 ± 3.22) ($P < 0.05$). However, the LG and BME groups did not differ from each other in terms of DNA damage ($P > 0.05$).

The values of the extender groups per treatment group were also examined statistically (Table 4 and Fig. 6a), and no significant differences were found among the extender groups within the C, P, and R treatment groups ($P > 0.05$); however, there were significant differences among the extender groups within the PR treatment group ($H = 7.66$; $df = 2$; $P < 0.05$). Higher level of DNA damage was observed in the C group (9.88 ± 0.75) compared with those in the LG and BME groups within the PR treatment group ($P < 0.05$), but the levels of DNA damage in the LG and BME groups were almost same (5.88 ± 1.11 and 6.25 ± 0.65, respectively) ($P > 0.05$). Although DNA damage values of the extender groups within each of the C, P, and R treatment groups did not differ statistically, supplementation of LG or BME to the extender is likely to decrease the level of DNA damage because relatively decreases in DNA damages were observed in the LG and BME groups compared with that in the C group within these treatment groups.

3.2.2. TB staining

The percentages of the C, LG, and BME extender groups were

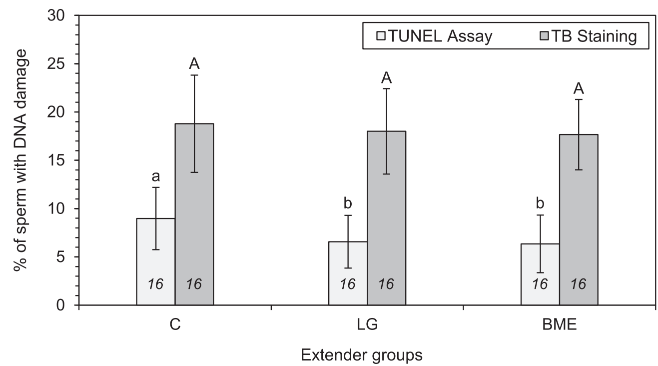

Fig. 5. DNA damages detected in the extender groups using the TUNEL assay and the TB staining. Numbers in the columns show n values (the numbers of aliquots). Means that do not share a letter in each method are significantly different from each other. C: Control group, LG: L-Glutamine group, BME: Basal medium eagle group.

18.78 ± 5.03, 18.00 ± 4.43, and 17.66 ± 3.63, respectively (Table 3 and Fig. 5). Extender groups were examined statistically, and it was found that there were no significant differences among them ($P > 0.05$). Though the case is not statistically significant, relatively high level of DNA damage was observed in the C group compared with the LG and BME groups.

The values of the extender groups per treatment group were also examined statistically (Table 4 and Fig. 6b), and no significant differences were found among them ($P > 0.05$). Although not statistically significant, the supplementation of LG or BME to the extender is likely to decrease the level of DNA damage in some treatment groups (LG: in the C and P treatment groups; BME: in the P and PR treatment groups).

3.3. Method comparison and correlation

The two methods used in this study to detect the number of sperm cells with DNA damage were analysed, it was determined that there was statistically significant difference between them ($P < 0.001$). Number of the sperm cell with DNA damage obtained using the TB staining was higher (18.15 ± 4.33) compared with that obtained using the TUNEL assay (7.29 ± 3.15). The pattern was the same when all treatment and extender groups were assessed.

A moderate correlation was found between the TUNEL assay and the TB staining ($r^2 = 0.54$; $P < 0.001$). Accordingly, there was a positive correlation between the percentage of TUNEL-positive sperm cells (percentage of DNA fragmentation) and the percentage of TB-positive sperm cells (percentage of abnormal DNA integrity) (Fig. 7).

4. Discussion

The optimum cryopreservation requires both preserving the viability and motility of the spermatozoa and maintaining their metabolic function [47]. Freezing–thawing procedures of cryopreservation result in the generation of ROS in sperm samples

Table 3
Descriptive statistic data of the extender groups for the TUNEL assay and the TB staining. n values indicate the number of aliquots in each extender group.

Extender group	TUNEL assay			TB staining		
	n	Mean (%)	Range (%)	n	Mean (%)	Range (%)
C	16	8.97 ± 3.22^a	3.50–15.00	16	18.78 ± 5.03^a	11.50–26.00
LG	16	6.56 ± 2.73^b	3.00–13.00	16	18.00 ± 4.43^a	12.00–24.50
BME	16	6.34 ± 2.98^b	1.50–11.50	16	17.66 ± 3.63^a	10.50–24.50

Means that do not share a superscript letter within same column are significantly different from each other.

Table 4
Descriptive statistic data for the extender groups based on treatment groups for the TUNEL assay and the TB staining. *n* values indicate the number of aliquots in each treatment group.

Treatment group	Extender group	TUNEL assay			TB staining		
		n	Mean (%)	Range (%)	*n*	Mean (%)	Range (%)
C	C	4	7.63 ± 1.18^a	6.00–8.50	4	16.88 ± 1.25^a	15.50–18.50
	LG	4	6.13 ± 1.49^a	4.50–8.00	4	14.75 ± 1.55^a	12.50–16.00
	BME	4	4.50 ± 3.03^a	1.50–8.00	4	17.13 ± 1.75^a	15.00–19.00
P	C	4	13.13 ± 1.75^a	11.00–15.00	4	24.88 ± 1.11^a	23.50–26.00
	LG	4	10.38 ± 1.80^a	9.00–13.00	4	23.38 ± 1.31^a	21.50–24.50
	BME	4	10.38 ± 1.03^a	9.00–11.50	4	21.75 ± 2.66^a	19.00–24.50
R	C	4	5.25 ± 1.32^a	3.50–6.50	4	13.00 ± 1.08^a	11.50–14.00
	LG	4	3.88 ± 0.75^a	3.00–4.50	4	13.38 ± 1.25^a	12.00–15.00
	BME	4	4.25 ± 1.26^a	2.50–5.50	4	13.13 ± 1.80^a	10.50–14.50
PR	C	4	9.88 ± 0.75^a	9.50–11.00	4	20.38 ± 4.50^a	15.00–25.00
	LG	4	5.88 ± 1.11^b	4.50–7.00	4	20.50 ± 1.68^a	18.50–22.50
	BME	4	6.25 ± 0.65^b	5.50–7.00	4	18.63 ± 1.11^a	17.50–20.00

Means that do not share a superscript lower-case letter within same treatment group are significantly different from each other.

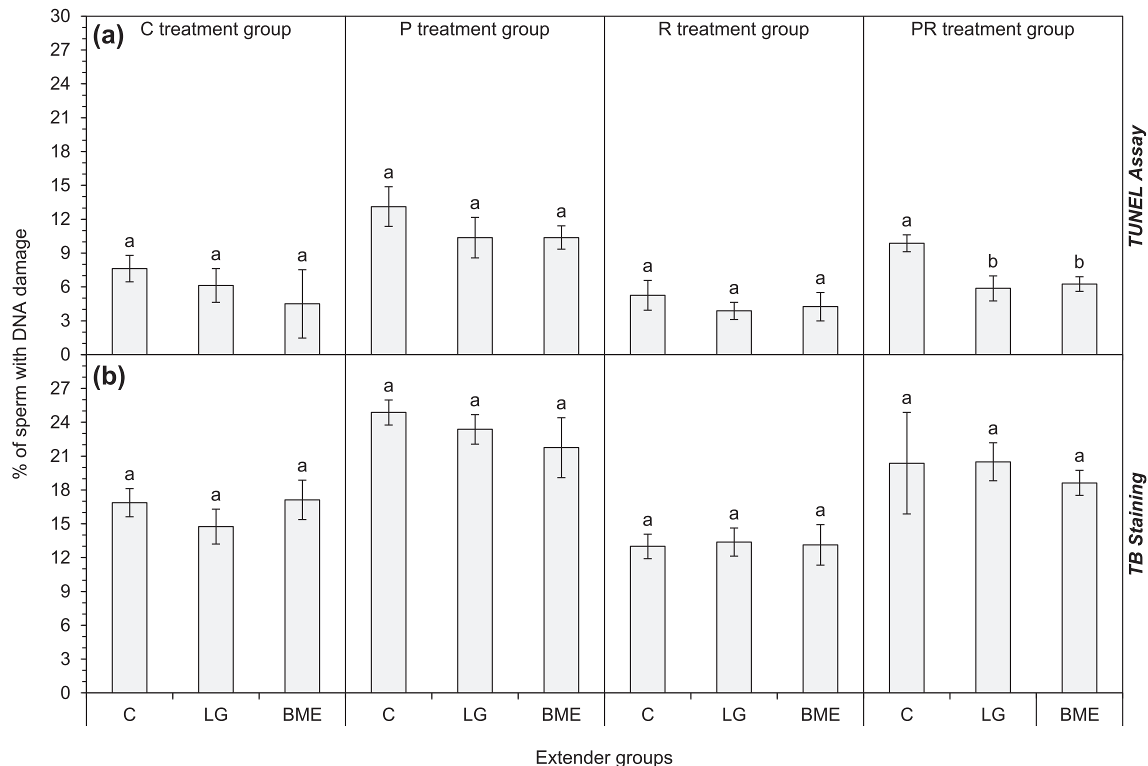

Fig. 6. DNA damages detected in the extender groups based on treatment groups using TUNEL assay (a) and TB staining (b). *n* value (the number of aliquots) for each extender group was four. Means that do not share a lower-case letter in each treatment group are significantly different from each other. Extender groups; C: Control group, LG: L-Glutamine group, BME: Basal medium eagle group. Treatment groups; C: Control group, P: Paclitaxel group, R: Resveratrol group, PR: Paclitaxel + Resveratrol group.

[48,49], and ROS diminish sperm motility, membrane integrity, and fertilising potential [48]. The cryoprotectants are added to extenders to protect the sperm from damage during freezing process [50]. The amount and type of cryoprotectants in semen diluent affect their effects on the sperm cells during freezing [51] and the achievement of the cryopreservation. As well as the sperm parameters mentioned above, DNA damage is of great importance for assisted reproductive technologies, and the evaluation of spermatozoa DNA damage can provide important data on the quality of spermatozoa and reproductive potential [52]. The etiology of DNA damage in the germ line has been reported to consist of three factors: oxidative stress, deficiencies in natural processes such as chromatin packaging and abortive apoptosis [53]. Sperm DNA fragmentation is an important parameter to assess sperm quality and can be considered as a putative fertility predictor [54]. Thus, researchers usually investigate the level of DNA damage in sperm samples after cryopreservation. In this study, we evaluated the levels of DNA damage of rabbit epididymal sperms after cryopreservation using the TUNEL assay and the TB staining methods and assessed the effects of paclitaxel, resveratrol, LG, and BME on DNA damage.

Paclitaxel is a chemotherapeutic used in the treatment of various cancer types [7]. Paclitaxel has been found to significantly induce DNA damage in the tests of genotoxicity on human lymphocytes [8,55]. It has been suggested that DNA damaging effect of paclitaxel might be biologically relevant as an alternative

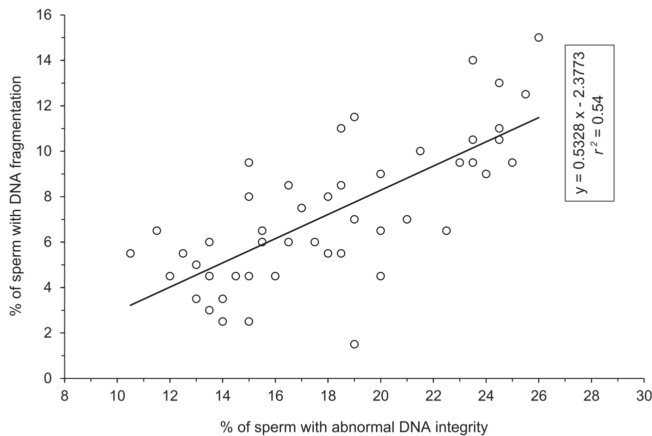

Fig. 7. Correlation between the percentages of DNA fragmentation caused by nicks and double-strand breaks (detected using the TUNEL assay) and abnormal DNA integrity (detected using the TB staining).

mechanism of tumor cell killing [56]. It has been observed that a reduction in spermatogonia population occurs in rats to whom paclitaxel is injected at maximum tolerated dose [57]. Similarly, it has been also reported that intraperitoneal administration of paclitaxel (5 and 4 mg/kg/week; for 10 weeks) causes a significant increase both in apoptotic germ cell number and in sperm DNA fragmentation in male adult rats [58]. In the present study, paclitaxel injection to the rabbits induced both DNA fragmentation and abnormal DNA integrity in sperm, agreeing with the results of Branham et al. [8], Digue et al. [55], and Sariözkan et al. [58]. It is well known that chemotherapeutic agents act by preventing rapidly proliferating cells, hence exerting their gonadotoxic effect. The extent of damage to germ cells and consequent fecundity depends on the class of chemotherapeutic agent, dosage, spermatogenetic stage targeted as well as the original pretreatment fertility potential of the patient [6,59]. Given these facts, paclitaxel might have had some other reverse effects on epididymal sperms in our study, but we evaluated only the level of DNA damage. Thus, it can be suggested that further studies should be carried out to reveal comprehensive results about the effects of paclitaxel on the sperms and the doses enabling paclitaxel to show these effects.

Resveratrol has been demonstrated to have antioxidant [60,61], antiinflammatory [60,62], antiproliferative [11,12] and scavenging activities [63], which may also be beneficial in preventing adverse environmental effects on spermatogenesis. It has been shown that the addition of resveratrol to semen prevents sperm DNA damages both in fertile and infertile men [64] and that resveratrol injection prevents sperm from DNA damage in mice [65]. It has been, therefore, suggested that resveratrol could be considered in cryopreservation procedures to avoid or minimise DNA damages and preserve sperm integrity [64]. In the present study, resveratrol injection reduced both DNA fragmentation caused by double-strand breakage and abnormal DNA integrity in sperm which was caused by paclitaxel and occurred spontaneously. This result that shows the DNA damage reducing effect of resveratrol is parallel to those of Branco et al. [64] and Revel et al. [65]. However, the effects of resveratrol on the other sperm parameters that were not assessed here should be comprehensively studied and revealed particularly for the administration of resveratrol and paclitaxel in combination because administration of them in combination may cause more serious toxicity than administration of each drug alone. Given the fact that cryopreservation may cause damages to the spermatozoa, probably due to the production of ROS, and that paclitaxel has DNA damaging effect as well as other effects on the sperm samples, it is clear that resveratrol is protective and recuperative material in the assisted reproductive technologies as an antioxidant. Although resveratrol is generally added to sperm samples just before freezing, we can suggest that IV injection of resveratrol is also beneficial for cryopreservation particularly in terms of DNA damage caused by chemotherapeutic agents such as paclitaxel. In addition, considering the DNA damage reducing effect of resveratrol demonstrated here, the concentration of resveratrol used in the present study is an effective concentration to be assessed as an antioxidant agent.

A wide variety of cryoprotectants are used in cryopreservation of the sperms of different organisms to protect them from damaging effects of low temperatures. In some organisms, amino acids have a protective effect against low temperatures [41]. Some researchers have reported that several amino acids protect spermatozoa against cold shock by forming a layer on the plasma membrane of the spermatozoon [66,67]. It has been reported in a study by Bucak et al. [37] that LG supplementation with a concentration of 5 mM in semen extenders improved post-thaw motility and membrane integrity in ram sperm and hereby provided a cryoprotective effect. In another study, it has been assumed that 80 mM glutamine can protect human spermatozoa during the freezing-thawing process and improve the motility and fertility capacity of post-thawed sperm [68]. On the other hand, no significant differences has been observed for DNA damage of frozen-thawed buffalo spermatozoa in extender containing glutamine (15 mmol) [69]. In the present study, it was found that LG in the extender seemed to reduce DNA fragmentation and abnormal DNA integrity levels, since decreases in DNA damage levels were observed both in general and in the treatment groups (see Figs. 5 and 6), aggreeing with the results of Bucak et al. [37] and Renard et al. [68]. The result obtained in this study implies that LG supplementation to the extender provides a cryoprotective effect against DNA damages. Taking all these results into account, we can suggest here that LG can be considered as an effective cryoprotectant to prevent DNA damages for freezing process of sperm samples, but nevertheless it can be speculated that the concentration of LG used in the study (5 mM) seems to be low due to the statistical exceptions in some of the groups, and hence further studies are needed to reveal the optimal concentration of LG for protecting the cryopreserved sperm samples from DNA damages.

Amino acid solution, namely BME, has been recently studied for its protective effect against low temperatures. Bucak et al. [37] have reported that BME supplementation to the extender at two different doses (13 and 26%) is not effective in the prevention of malondialdehyde formation, as indices of lipid peroxidation in the ram sperm samples. Similarly, Gungor and Bucak [41] have stated that BME added to extender (2.5 and 10%) may improve the post-thawed sperm parameters but not DNA integrity on freezing of Kangal dog semen. In the current study, it was determined that BME in the extender seemed to reduce DNA fragmentation and abnormal DNA integrity levels, since decreases in DNA damage levels were observed both in general and in the treatment groups (see Figs. 5 and 6). The result obtained in this study implies that BME supplementation (2.5%) to the extender provides a cryoprotective effect against DNA damages. The differences in the results of these studies may due to differences in the investigated species, extender composition, and antioxidant dose. Although this is the case, different and higher concentrations of BME should be tested for their cryoprotective effects on DNA damage and be detected the optimal concentration to use for cryopreservation of epididymal sperms. In addition, because BME consists of 13 amino acids and eight vitamins [36], one can expect that it provides more effective protection against freezing-thawing process than LG in terms of DNA damage. Indeed, BME resulted in lower levels of DNA damage

in the majority of group evaluations compared with LG in this study, and this is consistent with the mentioned expectation.

Different methods are currently used for studying abnormalities in the sperm DNA. Some of these methods are acridine orange (AO) staining, sperm chromatin structure assay (SCSA), COMET assay [70], and aniline blue (AB) staining [71]. Apart from these, the TUNEL assay, which allows the evaluation of the sperm DNA fragmentation [45,72,73], and the TB staining, which identify the packaging defects of sperm chromatin [46,71] by detecting the absence or rupture of disulfide bonds, have been used with the aim of studying sperm DNA status for many decades. The correlation between the TUNEL assay and the TB staining methods obtained in this study shows the linkage between DNA fragmentation and abnormal DNA integrity, meanly chromatin compaction. In addition, the fact that the detected correlation was positive indicates the level of DNA fragmentation to increase with the increasing level of abnormal DNA integrity. The level of abnormal DNA integrity can be considered to demonstrate the susceptibility of DNA to fragmentation, hence that the level of abnormal DNA integrity was higher than that of DNA fragmentation is an expected result. There have been some studies reporting that the TB staining shows high correlation coefficient with the TUNEL assay (i.e., Erenpreiss et al. [74]). It is noteworthy that a relatively high correlation coefficient was also found between the TUNEL assay and the TB staining in the present study. It has been well documented that sperm DNA damage affects the fertility outcome, but there is no consensus on which technique/techniques to use DNA evaluation in the sperm samples. The methods used to investigate sperm DNA damage should be standardised to allow comparison among different studies and to permit routine use of tests in clinical laboratories. Given these facts, it can be stated that the TUNEL assay and the TB staining methods are simple, less expensive procedures that are performed in a short time to assess the levels of DNA damage in the sperm samples.

Accurately detecting biochemical, metabolic, and genetic defects that may occur in sperm and may decrease its fertilisation capability is of great importance not only in veterinary but in human andrology. Flow cytometry is an extremely useful and robust tool that can be used for this purpose because it provides quick and accurate results, enabling an accurate estimation of various sperm parameters such as mitochondrial activity, oxidatif stress, DNA status, acrosome integrity, and sperm count [75,76]. A number of characteristics of sperm integrity, viability, and function can be assessed using flow cytometry. It is considered as a tool that may be used in the future to monitor many new potential markers of sperm function with the growing technology [75].

5. Conclusions

In the present study, paclitaxel as a chemotherapeutic agent resulted in higher levels of DNA fragmentation and abnormal DNA integrity in the rabbit epididymal sperms, but resveratrol ameliorated this effect of paclitaxel and reduced the levels of both DNA damages. Several aspects of our results provide compelling evidence that LG and BME supplementation to the extender prevented the sperm samples from DNA damage after cryopreservation and these additives with the correct doses would yield a good cryoprotection in terms of DNA damage. It was determined that the TUNEL assay was positively correlated to the TB staining and the level of abnormal DNA integrity was higher than that of DNA fragmentation, showing that the TB staining demonstrates the susceptibility of DNA to fragmentation in the sperm, but the TUNEL assay demonstrates the fragmented DNA. We conclude that chemotherapy drugs cause the sperm DNA to be damaged, and hence adversely affect the fertility of male cancer patients of reproductive age. The administration of resveratrol together with paclitaxel may ameliorate the DNA damage inducing effect of paclitaxel, but the effects of the administration of resveratrol and paclitaxel in combination on the other sperm parameters should be necessarily studied, since the administration of them in combination may cause more serious toxicity than the administration of each drug alone. On the other hand, sperm banking can be suggested to all male cancer patients of reproductive age facing cancer treatment for fertility preservation. Cryopreservation of their sperms with the appropriate cryoprotectants such as LG and BME prior to cancer treatment can guarantee their fertility after the treatment. This and suchlike studies are of paramount importance not only for the conservation and the persistence of endangered animal species but also for the reproductive results of cancer treatment and the prevention of these results after the treatment.

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