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Effects of centrifugation and washing of freeze-thawed blood on isolated DNA characteristics

Mevlüt ARSLAN*
Department of Genetics, Faculty of Veterinary Medicine, Van Yüzüncü Yıl University, Van, Turkey

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Abstract: DNA isolations from the whole blood are commonly performed to obtain DNA for molecular research and diagnostics. Generally, blood samples are taken into anticoagulant tubes and stored in deep freezers until DNA isolation. In fresh blood, pre-treatments or leukocytes preparations can be performed and suggested for advanced DNA isolation. However, similar applications in freeze-thawed blood (FTB) have not been shown yet. In the study, centrifugation and washing of FTB were applied as pretreatment before DNA isolations, and their effects on isolated DNA characteristics including DNA integrity, quality, quantity, mitochondrial (mt), and nuclear (n) DNA levels were investigated. Microscopic and flow cytometric analyses were used to check leukocyte integrity in FTB. Spectrophotometric analysis was carried out to determine DNA quality and quantity in the isolated DNA samples. Real-time PCR analyses were used to check mtDNA/nDNA ratio and DNA integrity at the quantitative level. Cell integrity analyses showed that most of the leukocytes were intact in FTB. Therefore, centrifugation enabled intact leukocytes and nuclear pellets in FTB to be harvested and washed and could be applied as pretreatment before DNA isolations. PBS and water washing of FTB led to obtaining high-quality DNA without changing the nDNA/mtDNA ratio and DNA integrity. TE washing of FTB increased DNA quality and enriched nDNA level about 2-fold without changing DNA integrity. Centrifugation and harvesting of a higher volume of FTB increased isolated DNA yield and quality but decreased DNA integrity and nDNA level. To conclude, the pretreatments of FTB had the advantage to obtain DNA with high-quality and high-quantity and can be used before DNA isolation, but they may affect mtDNA/nDNA ratios and DNA integrity levels. The relevant pre-treatment used in the present study can be used and improved for desired DNA isolation from FTB samples.

Key words: Blood, freeze-thawing, DNA isolation, centrifugation, washing, DNA quality

1. Introduction

In molecular research or diagnostics, obtaining DNA from blood is a commonly used application all around the world [1–3]. Erythrocytes, leukocytes, and thrombocytes are the main cell types in mammalian blood. Leukocytes are the main resource of isolated DNA in the mammalian blood. Even though erythrocytes comprise about 99% of whole cells in the blood, they lack genomic DNA. Therefore, the main concern in DNA isolations from blood samples is leukocytes [4–6].

Fresh blood or FTB can be used as a source in DNA isolations. Using fresh blood is advantageous due to leukocyte preparations. In fresh blood, two main approaches are applied to separate leukocytes. The first one is density gradient centrifugation of whole blood, which leads to obtaining a buffy coat [7,8]. The buffy coat contains leukocytes and DNA isolation can be performed from the buffy coat [9]. The second one to separate leukocytes is selective lysis of erythrocytes. Erythrocyte or red blood cell (RBC) lysis solutions disrupt mainly erythrocytes, while leukocytes remain intact. Then, centrifugation leads to the harvesting of leukocytes called white cell pellets [10]. DNA isolation performed from leukocyte preparations is advantageous compared to whole blood [3, 11, 12]. In practice, blood samples are taken into vacutainers with ETDA and stored in deep freezers until DNA isolation. Since freeze-thawing of whole blood lyses blood cells, leukocyte enrichment applications in FTB are not generally used [13–15]. However, in freeze-thawing of blood, leukocytes are not completely lysed [16] and nuclear pellets from the lysed cells can be harvested by centrifugation [17,18]. Therefore, it was hypothesized that centrifugation and washing of FTB might be applied for pretreatment in FTB for DNA extractions and might be used to obtain DNA with high-quality and high-quantity from the FTB specimens.

The aim of the present study was to evaluate the applications of centrifugation and washing of FTB as a pretreatment in DNA purifications in FTB samples and to determine their effect on isolated DNA characteristics including DNA integrity, quantity, quality, mtDNA/nDNA ratios. Cell integrity analysis was carried out to show and

* Correspondence: mevlutarslan@yyu.edu.tr

determine leukocytes lysis level in FTB. Centrifugation and washing of FTB enabled intact and lyzed leukocytes in FTB to be harvested and washed, and they were used as pretreatments of FTB before DNA isolations. The isolated DNA characteristics were studied in detail. Real-time PCR studies shed light on the DNA integrity and mtDNA/nDNA ratios in the isolated DNA samples, while spectrophotometric analyses highlighted DNA quantities and qualities.

2. Materials and methods

2.1. Blood sampling

Bovine whole blood samples were collected in vacutainer with EDTA and stored at –20 °C or 4 °C for staining, flow cytometry, and DNA isolations. Blood samples at 4 °C were studied immediately. This study was approved by the Animal Researchers Local Ethics Committee of Van Yüzüncü Yıl University (Approval 07.03.2019, 2019/2).

2.2 Cell integrity

In FTB, cell integrities were determined by microscopy and flow cytometry. Simple staining was used preliminarily. For this purpose, Giemsa staining was carried out in the fresh blood, FTB, and pellet obtained from FTB and examined in light microscopy. Photographs were taken by using Nikon Eclipse E400, a Y-THM imaging system.

Flow cytometry was also used to evaluate cell integrity in FTB. For this purpose, blood samples were taken into vacutainer tubes with EDTA and frozen in a deep freezer (–20 °C). On the day of flow cytometry analysis, frozen blood samples were thawed at room temperature, and new fresh blood samples were collected in the blood tubes containing EDTA and stored at 4 °C and studied immediately. For flow cytometry analysis, the fresh and FTB samples were lysed using 1x RBC lysis solution. Briefly, 10x RBC lysis solution (1.5 M Ammonium chloride, 100 mM sodium bicarbonate, 10 mM disodium EDTA, pH 7.4) was prepared and stored at 4 °C. Before use, it was diluted 1x concentration by using distilled water. A total of 100 µL volume of the fresh or FTB samples were added into 2 mL 1xRBC lysis solution, mixed gently, and incubated for 15 min. Then, the tubes were centrifuged at 500 *xg* for 5 min. Supernatants were discarded, and pellets were resuspended in PBS. Flow cytometry analysis was carried out by using BD LSR Fortessa, and 100.000 cells were read in each tube (n = 5).

2.3. DNA isolation

Frozen blood samples were thawed at room temperature. In the control group (called as Control), DNA isolation was carried out by using 200 µL (one-volume) FTB and following the commercial DNA isolation kit's protocol (Vivantis GF, Malaysia). In the experimental groups, two main strategies were followed: direct centrifugation of FTB and centrifugation and/or washing of FTB (Figure 1). For this aim, five different experimental groups were set up. In the first group (called Pellet200), centrifugation level (10.000 × *g* for 1 min) was checked to harvest leukocytes and lysed nuclear pellets in FTB. Also, in this group, the effect of reduction of hemoglobin and EDTA level on DNA isolation was investigated. For this purpose, 200 µL (one-volume) of FTB samples were added into microcentrifuge tubes and centrifuged at 10.000 × *g* for 1 min. Then, 150 µL supernatant was discarded and 150 µL distilled water was added into the remaining part to obtain 200 µL total volumes. The following applications were performed as described in the manufacturer's protocol. In the second group (called Pellet400), to obtain a high amount of DNA, centrifugation of higher volumes of FTB was check to enrich genetic material, For this aim, 400 µL (two-volume) of FTB was added into the microcentrifuge tubes and centrifuged at 10.000 × *g* for 1 min, and 350 µL of the supernatant was discarded, and 150 µL distilled water was added to obtain 200 µL total volumes. The following applications were performed according to the manufacturer's protocol. In the last three groups, washing of pellets obtained from FTB was carried out. Washing applications were performed to eliminate hemoglobin and EDTA from leukocytes and nuclear pellets, and also to investigate the washing effect on isolated DNA integrity. TE washing (10 mM Tris-HCl,

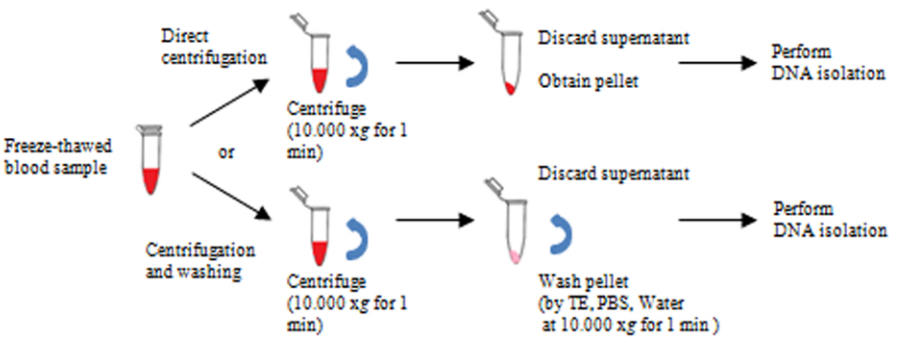

Figure 1. The schematic representation of two main strategies followed in this study.

1 mM EDTA, pH 8), which is a commonly used buffer to protect DNA from nucleases, was applied to check any nuclease degradation of DNA during washing. In the TE washing group (called TE), 200 µL FTB was added into microcentrifuge tubes. Then, 200 µL TE was added into the tubes and vortexed, and centrifuged at 10.000×*g* for 1 min. Then, 200 µL of the supernatant was discarded and 200 µL TE was added into the tubes and vortexed and centrifuged at 10.000 × *g* for 1 min. This procedure was performed three times, and pellets were washed by TE. In the last two groups, commonly used washing solutions, phosphate buffer solution (PBS), and distilled water were used to wash leukocytes and nuclear pellets in FTB. The same application performed for TE washing was carried out for PBS (called PBS) and distilled water (called Water) washing.

2.4 DNA quality and quantity

Isolated DNA samples were analyzed spectrophotometrically by using Nanodrop2000c (Thermo Scientific, USA). DNA concentrations (ng/µL), A_{260}/A_{280} ratios, and A_{260}/A_{230} ratios in the isolated DNA samples were determined. DNA samples were stored at –20 °C for the following analyses.

2.5. Determination of nDNA/mtDNA ratio

To investigate the effect of centrifugation and washing of FTB on nDNA/mtDNA levels in the isolated DNA samples, quantitative real-time PCR (qPCR) was used. In the qPCR analysis, the basic transcription factor 3 (*BTF3*) gene, a copy number reference gene, was used to eliminate individual copy number differences [19–21]. Similarly, for the investigation of mtDNA levels in the isolated samples, the *ND1* gene was chosen [22]. To design primers of interest, the online Primer-Blast tool was used [23]. Selected primers were shown in Table 1. Self and hetero-dimer properties were checked as described previously [24].

qPCR analysis was performed with Steponeplus real-time PCR system (Applied Biosystem). PCR cycling conditions were as follows: a preincubation phase of 5 min at 95 °C was followed by up to 40 cycles of 3 s at 95 °C, 30 s at 58 °C. The reaction mix included 1xAmplifyMe Syber Green Master Mix (Blirt, Poland), 0.3µM primer concentration of each primer, and 1 µL of isolated DNA samples. Melting curve analysis was performed by increasing temperature gradually (0.3°C/10s) from 60 °C to 95 °C. To compare mtDNA and nDNA levels in the control group and experimental groups, the 2ˆ(–delta delta CT) method was performed [25].

Table 1. Primers used in the mtDNA and nDNA ratio analysis.

Gene	Region	Direction	Sequence (5'->3')
ND1	mtDNA	Forward	AGCCATATCAAGCCTAGCCG
		Reverse	TTTGAGTTGGAAGCTCAGCC
BTF3	nDNA	Forward	CATGTCCTACACAGGCGAAG
		Reverse	GAAATTCGGGAGCTTGGCGG

2.6. DNA integrity

DNA integrity in the isolated DNA samples was assessed quantitatively by long-run real-time PCR as described previously [26]. In the long-run real-time PCR, the optimized mix consisted of 1 U Hot FIREPol DNA Polymerase (Solis Biodyne, Tartu, Estonia), 2 mM $MgCl_2$, 1× buffer B1, 200 µM dNTPs (Solis Biodyne, Tartu, Estonia), 2 µM SYTO82 (Life Technologies, USA), primers at a concentration of 500 nM (Table 2) and ~50 ng of DNA template in a 20 µL reaction volume. To calculate PCR efficiencies, 50 ng, 25 ng, 12.5 ng, 6.25 ng standard template dilutions were used. The copy number reference gene, *BTF3*, was chosen for PCR amplification. Primers were designed by using the online Primer-Blast tool [23], and self and hetero-dimer properties were checked as described previously [24].

PCR cycling conditions were as follows: a preincubation phase of 15 min at 95 °C was followed by up to 50 cycles of 15 s at 95 °C, 15 s at 60 °C, and 240 s at 72 °C for long amplicons. For small amplicons, PCR cycling conditions were as follows: a pre-incubation for 15 min at 95 °C was followed by up to 40 cycles of 5 sec at 95 °C, 15 s at 60 °C and 1 s at 72 °C. In real-time PCR, the second channel was used for fluorescence readings (LineGene K plus, Bioer). Amplicons' specificities were checked by melting curve analysis and agarose gel electrophoresis. DNA integrities

Table 2. Primers used in the long-run real-time PCR.

Primers	Sequence (5'>3')	PCR product (bp)
Long Forward	GGCAATACCAGTTGAATTTG	3067
Long Reverse	TGTGTTAGTACGTTCCTAGT	
Short Forward	TTTTATACTCTCAGGATTGAGC	70
Short Reverse	TTGATATTTTCTTCAGGTACACTAT	

(DNA lesion per 10 kb) were calculated in the following previously described equation [26].

2.7. Statistical analysis

The student's t-test (two-way, unpaired) was used to calculate significance levels in experimental groups compared to the control group. Statistical analyses were performed on R software using the 'stats' package (R Development Core Team 2017) [27]. Data points in figures corresponded to mean and standard deviations ($n \geq 3$).

3. Results

Simple staining shed light on the cellular state in the fresh blood, FTB, and pellet obtained by centrifugation of FTB (Figure 2). In the fresh blood, blood cells were shown their characteristic structures (Figure 2a). In the FTB, most of the cells were shown to be lysed (Figure 2b). In the pellet obtained by centrifugation of FTB, intact cells with different morphologies or nuclear debris were shown (Figure 2c). Centrifugation of FTB enabled lysed and intact leukocytes to be collected in pellets, which led to advantageous DNA isolation.

Flow cytometry analysis highlighted deeply the effect of freeze-thawing on blood cells (Figure 3). In the fresh blood, leukocytes were separated well into the subpopulations (Figure 3a). However, in FTB, leukocyte populations were not separated, and the cell debris population increased (Figure 3b). This result clearly indicated that freeze-thawing of blood affected cellular morphology and subcellular state without lysing leukocytes. Although freeze-induced lysis occurred, most of the leukocytes were intact.

Spectrophotometric analysis results highlighted the effect of centrifugation and washing applications on the isolated DNA quantities and qualities (Figure 4). Using a higher volume of FTB and harvesting by centrifuge increased DNA yield about 2-fold (Pellet400), which might be a key application to the desired high quantity DNA in per isolation (Figure 4a). More interestingly, total DNA yield decreased about 2-fold in TE washing of FTB. However, PBS and water washing of FTB did not affect the DNA yield. In all tested groups, the A_{260}/A_{280} ratios of the isolated DNA were almost similar. Even though the A_{260}/A_{280} ratios of the PBS group seemed to be different, the ratio was at the desired level (1.9 ± 0.01) (Figure 4b). A_{260}/A_{230} ratios of isolated DNA samples increased significantly in all tested groups compared to the control group (Figure 4c). A_{260}/A_{230} ratios in the all tested group were at the desired level (2-2.2), whereas the ratio was low in the control group [28], which indicates that all applications increase isolated DNA quality.

mtDNA and nDNA levels in the isolated DNA samples were investigated by real-time PCR analysis (Figure 5). Washing of pellet by PBS and water did not affect mtDNA/nDNA ratios compared to the control group. Interestingly, TE washing of FTB led to the enrichment of nDNA level in isolated DNA samples, which suggests that TE washing of FTB might cause mtDNA loss (Figure 5a). Furthermore, direct centrifugations of FTB (one-volume or two-volume) led to the enrichment of mtDNA (Figure 5b).

Long-run real-time PCR highlighted the DNA integrity levels in the isolated DNA samples (Figure 6). The results indicated that all washing applications of FTB did not affect DNA integrity. However, DNA integrity was found to be lower in the pellets of one-volume and two-volume of FTB samples.

4. Discussion

Until now, no study has used centrifugation and/or washing of FTB before DNA isolation and reported their effect on isolated DNA characteristics. The present study first reports the effects of centrifugation and washing of FTB on the isolated DNA characteristics including DNA integrity, quantity, quality, and mtDNA/nDNA ratios.

It is known that freeze-thawing without cryoprotectants lyses mammalian cells. Due to the lysis of blood in freeze-thawing, pretreatments or leukocyte preparations in FTB were not used before DNA isolation. The primary effect of freeze-thawing on the cells is disruption of the plasma membrane [14,29]. Mitochondria, lysosomes, other organelles, and the nucleus are the other side of freezing injury in the cell [30–35]. It was suggested that plasma membrane

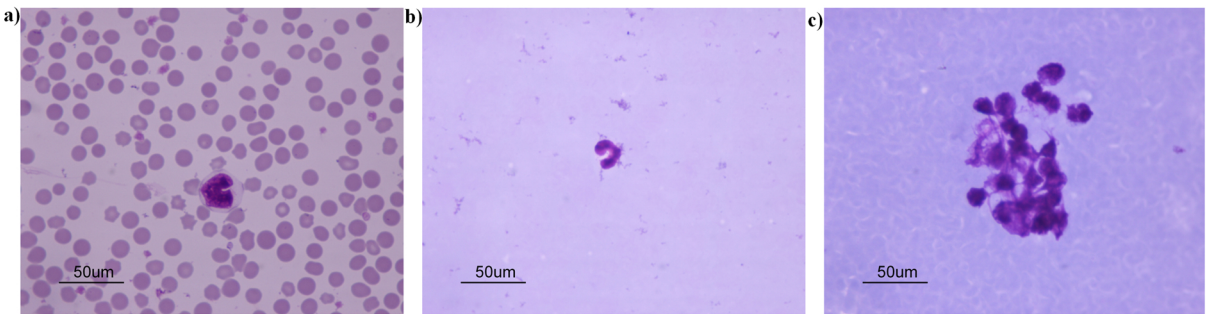

Figure 2. Microscopic view of fresh blood (a), freeze-thawed blood (b), and pellet of the freeze-thawed blood (c).

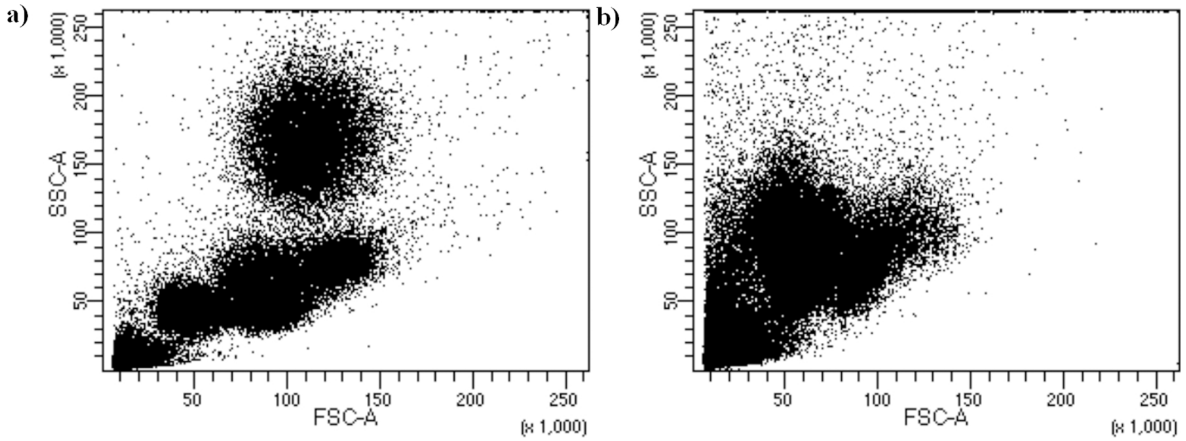

Figure 3. Flow cytometry analysis of fresh (a) and freeze-thawed (b) blood samples. A representative result is shown; the experiment was repeated five times, but similar results were obtained.

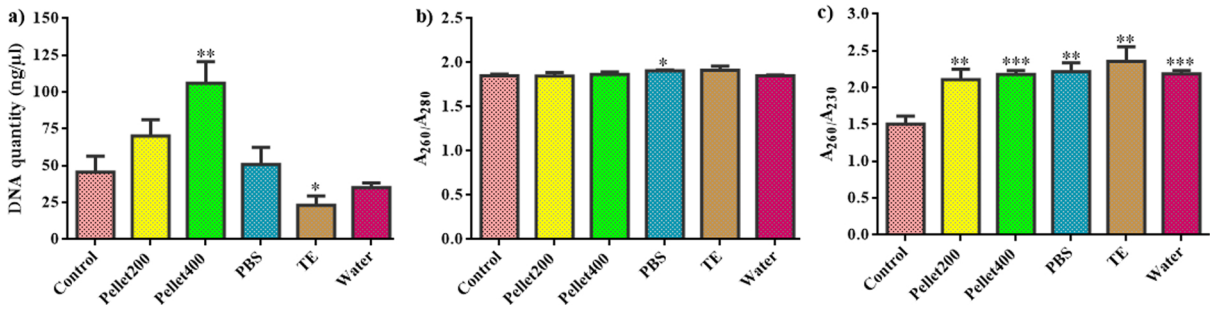

Figure 4: Quantities and qualities in the isolated DNA samples. (a) DNA concentrations, (b) A_{260}/A_{230} ratios, and (c) A_{260}/A_{280} ratios in the isolated DNA samples in each group. * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$.

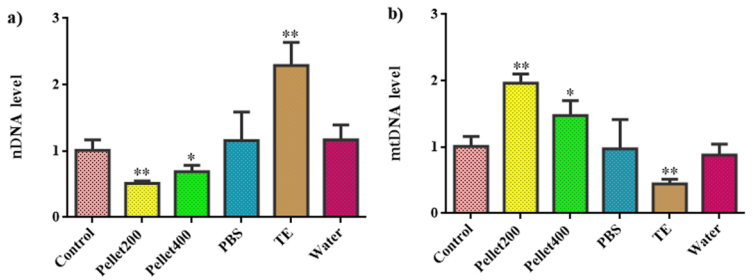

Figure 5. nDNA and mtDNA levels in the isolated DNA samples. nDNA level (a) and mtDNA level (b) are shown, which are obtained by $2^{-\Delta\Delta Ct}$ method based on $Ct_{TERT\text{-}ND1}$ and $Ct_{ND1\text{-}TERT}$, respectively. * and ** indicate $p < 0.05$ and $p < 0.01$ statistically significant levels, respectively.

was not the primary site of freeze injury when slow freezing took place [36,37]. Lippi [16] reported that freeze/thawing did not lyse leukocytes completely. In the current study, flow cytometric analysis revealed that most leukocytes were intact even though morphological changes were occurred (Figure 3). There was evidence that centrifugation of chemical-lized blood could harvest nuclear pellets [17, 18]. This could be due to the organization of nDNA by histone proteins called chromatin in the interphase cells [38–40]. This organization of nDNA leads to an increase in molecular weight; thereby, centrifugation enables organized nDNA in the lysed fraction to harvest in the pellet (Figure 2c). Therefore, intact leukocytes or organized DNA in lysed leukocytes in FTB can be centrifuged and washed before DNA isolations. This application can be used for the desired DNA isolations with high-quality and high-quantity.

DNA samples with high-quality and high-quantity are desired for successful downstream applications such

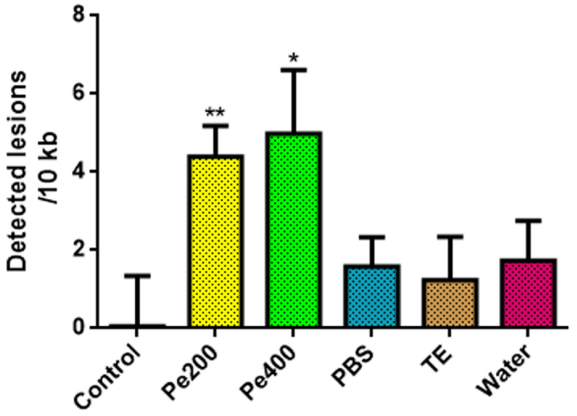

Figure 6. DNA integrity levels in the isolated DNA samples. * indicates $p < 0.05$, ** indicates $p < 0.01$.

as SNP genotyping, PCR, and sequencing [1,28,41–43]. Here, it was shown that harvesting two-volume of FTB by centrifugation led to obtaining DNA with higher quantity and quality by using the same materials with one-volume of FTB (Figure 4a). This could be due to harvesting more genomic sources and eliminating hemoglobin/EDTA in lysed blood. Interestingly, TE washing of FTB led to a decrease in the isolated DNA yield. TE might decrease the DNA binding properties to the silica column or increase the lysis of the freeze-thaw-injured leukocytes. For a high-quality DNA sample, A_{260}/A_{280} and A_{260}/A_{230} ratios should be ~1.8 and 2-2.2, respectively [28]. A_{260}/A_{230} ratio decreases in the presence of contaminants including EDTA, guanidine HCl (used for DNA isolations), lipids, carbohydrates, phenol, or salts [44]. Even though centrifugation and washing of the FTB had no significant effect on A_{260}/A_{280} ratios, these applications significantly increased A_{260}/A_{230} ratios, indicating contaminant-free DNA in all tested groups (Figure 3c). This might be due to the elimination of hemoglobin and EDTA found in the blood tubes at the beginning of DNA isolation with the pretreatments.

In general, DNA purifications from fresh or FTB, nDNA, and mtDNA, which compose total DNA, are isolated together,. When one target is required, the other DNA source is waste [45]. Otherwise, when a special study such as mtDNA copy number research has been carried out, mtDNA and nDNA levels are important [46–50]. Therefore, the effect of centrifugation or washing on mtDNA/nDNA ratios in the isolated DNA samples was checked. It should be expected that the mtDNA level might decrease due to ice-induced cell lysis and centrifugation [14, 29]. However, it was shown that mtDNA levels did not decrease in the centrifuge and washing groups (Figure 5b). Flow cytometry analysis shed light on this situation (Figure 3). It was shown that most of the cells were intact in FTB even morphological and subcellular differentiations occurred (Figure 2). This finding was also reported by Lippi [16]. Lippi reported that freeze/thawing did not lyse leukocytes completely. The remaining intact leukocytes in the pellet might be the source of mtDNA. Furthermore, direct centrifugation increased mtDNA levels in isolated DNA samples (Figure 5b). It is suggested that hemoglobin forms insoluble hemoglobin complexes and decreases the saturation of silica membrane, which results in decreasing DNA yield and purity. Decreasing hemoglobin concentration and EDTA in the tubes might increase membrane saturation, resulting in increased mtDNA capturing. Water and PBS washing of FTB did not affect the isolated mtDNA/nDNA levels (Figure 5). However, TE washing of FTB decreased the mtDNA levels in the isolated DNA, where nDNA enriched almost 2-fold (Figure 5a). It should be noted that TE washing of FTB resulted in almost two-fold decrease in DNA yield (Figure 4a). Therefore, it can be concluded that TE washing of FTB did not affect nDNA amount in total; however, it caused a decrease in the mtDNA level. This can be clearly shown in qPCR analysis (Figure 5). TE might affect the binding of mtDNA to the column membrane or cause lysis of freeze-injured leukocytes in FTB, leading to loss of mitochondria during the TE washing steps.

Previous studies have shown that freeze-thawing causes double-stranded breaks (DSBs) in genomic DNA. An earlier study revealed the marginated chromatin patterns and nuclear ice crystals in the frozen cells [51]. Trusal et al. [35] showed chromatin distribution and disruption in frozen (–20°C) bovine endothelial cells. It was reported that freeze-thaw cycles increased DNA degradation [52]. A recent study reported that the cryoprotectant dimethyl sulfoxide (DMSO) protected DNA from ice-induced DSBs [53]. Therefore, the effects of centrifugation and washing of FTB on isolated DNA integrity were investigated. It was shown that direct centrifugation of one and two-volume of FTB and elimination of supernatant (hemoglobin/EDTA e.g) lowered isolated DNA integrity (Figure 6). This could be due to the elimination of supernatant, where hemoglobin and EDTA levels decreased significantly. This might increase column binding and capture of the fragmented DNA. However, all washing applications of FTB did not affect isolated DNA integrity. In the washing steps, fragmented DNA could be lost, and thereby DNA integrity did not change.

A limitation of the study is that a commercial DNA isolation kit was used in the present study. Different DNA isolation kits could be used to check achieved results. However, a lot of current DNA isolation kits use column-based DNA isolation. In this study, a column-based DNA isolation kit was used, which indicates that similar results could be obtained.

In conclusion, centrifugation and washing of FTB can be used to wash and harvest intact leukocytes and lysed nuclear pellet in FTB as a pretreatment in DNA purifications. These applications can be used to obtain high-quality and high-quantity DNA samples. When mtDNA/nDNA levels and DNA integrity are considered, a relevant application described in the study can be used and improved in light of the current study.

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