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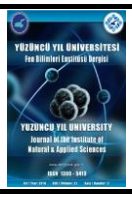

Research Article

Effects of Centrifugation at Different Levels of Freeze-Thawed Blood on DNA Isolation

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Abstract: DNA isolation from blood is a commonly used application to obtain nDNA and mtDNA. It was previously shown that DNA isolation could be performed from the pellet obtained after centrifugation of freeze-thawed blood (FTB), and this pretreatment had constructive results on DNA isolation. However, which centrifugation levels can be used for this pretreatment, and their effects are unknown. The aim of the study was to determine appropriate centrifugation levels for this pretreatment and show their effects on isolated DNA. For this purpose, DNA isolations were carried out from pellet and supernatant obtained by centrifugation at different levels of FTB. Then, spectrophotometric, gel electrophoresis, and real-time PCR analyses were performed in the isolated DNA samples. As a result, centrifugation of FTB at 5,000×g for 2 min or over let genetic material to pellet completely. This also caused to obtain high amount of DNA. mtDNA/nDNA ratios did not change in the isolated DNA samples from pellets obtained by defined centrifugation levels, but the DNA integrity decreased. To conclude, centrifugation of FTB at 5,000×g for 2 min or over can be used to harvest and wash genetic material found in FTB before DNA isolations.

Donmuş Çözünmüş Kanın Farklı Değerlerde Santrifüjünün DNA İzolasyonu Üzerine Etkileri

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Anahtar Kelimeler
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Öz: Kandan DNA izolasyonu, nDNA ve mtDNA'yı elde etmek için yaygın kullanılan bir uygulamadır. Donmuş çözünmüş kanın (DÇK) santrifüjü sonrası elde edilen çökeltiden DNA izolasyonun gerçekleştirilebileceği daha önce gösterilmişti ve bu ön uygulama DNA izolasyonu üzerine olumlu sonuçlara sahipti. Fakat, bu ön uygulama için hangi santrifüj değerlerinin kullanılabileceği ve bunların etkileri bilinmemektedir. Bu çalışmanın amacı belirtilen ön uygulama için uygun santrifüj değerlerini belirlemek ve bu santrifüj değerlerinin izole edilen DNA üzerine ektilerini göstermekti. Bu amaçla, DÇK'nin farklı değerlerde santrifüjüyle elde edilen çökelti ve süpernatantdan DNA izolasyonları gerçekleştirildi. Sonra, izole edilen DNA örneklerinden spektrofotometrik, jel elektroforezi ve gerçek-zamanlı PCR analizleri gerçekleştirildi. Sonuç olarak, DÇK'nin, 5.000×g'de 2 dakika ya da üzeri değerde santrifüjü genetik materyali tamamen çöktürmeyi sağladı. Bu durum ayrıca yüksek miktarda DNA elde edilmesine imkan sağladı. Belirtilen santrifüj seviyelerinde elde edilen çökeltiden izole edilen DNA örneklerinde mtDNA/nDNA oranı değişmedi fakat, DNA bütünlüğü azaldı. Sonuç olarak, DÇK'nin 5.000×g'de 2 dakika ya da üzerinde santrifüjü, DNA izolasyonundan önce DÇK'de bulunan genetik materyali çöktürmek ve yıkamak için kullanılabilir.

1. Introduction

Obtaining nucleic acids from a biological specimen is the starting point for molecular research or diagnosis. Nucleic acid samples with high quantity and quality are essential for successful downstream molecular genetic studies. Therefore, nucleic acids extraction is an essential and primary step for these purposes (Chacon-Cortetes & Griffith, 2014; Hjorthaug et al., 2018). From Friedrich Miescher to today, a range of DNA extraction methods and techniques have been developed (Dahm, 2005; Chacon-Cortetes & Griffith, 2014; Green & Sambrook, 2018; Gautam, 2022). In these applications, mainly cells are lysed, nucleoprotein complexes are denaturized, nucleases are inactivated, non-desired biological and chemicals are eliminated, and lastly, DNA is precipitated (Tan & Yiap, 2009).

DNA extraction from blood samples is a widely used application for obtaining both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) (Chacon-Cortetes & Griffith, 2014). Blood samples are generally preferred in genetics, genomics, pharmacogenomics, and epidemiological studies (Visvikis et al., 1998; Gao et al., 2015; Lu et al., 2016; Rzehak et al., 2016). In these studies, both fresh and freeze-thawed blood (FTB) samples are used. Briefly, after sampling of blood, DNA isolation is not performed directly, and the samples are stored in deep freezers until processed (Bulla et al., 2016; Kaya et al., 2022). However, freeze-thawing without cryoprotectant has a detrimental effect on cell integrities (McGann et al., 1988; Steponkus & Lynch, 1989; Tansey, 2006). Therefore, leukocyte preparation processes are not generally performed. However, an earlier study reported that leukocytes were not completely lyzed in freeze-thawing of blood (Lippi, 2012). Furthermore, in a recent study, flow cytometry analysis showed that high amount of leukocytes were not lysed, even if subcellular changes occurred, in freeze-thawed (one cycle) blood (Arslan, 2022). Therefore, washing and centrifugation of FTB can be used to harvest and wash intact cells and nuclear pellet in FTB, and these pretreatments have constructive results on DNA isolation to obtain high-quantity and high-quality DNA from FTB (Arslan, 2022). However, it is not certain which centrifugation levels can be used for this application and how it affects isolated DNA characteristics such as mtDNA/nDNA ratio, DNA integrity, DNA quantity and quality.

This study aimed to determine the appropriate centrifugation level to harvest intact and lysed leukocytes for the pretreatment application and also investigate the effects on DNA quality, quantity, integrity, and nDNA/mtDNA rations in supernatants and pellets. For this purpose, DNA isolations were carried out from both pellet and supernatant obtained by centrifugation at different levels from freeze-thawed blood. Then, spectrophotometric, gel electrophoresis, and real-time PCR analyses were performed in the isolated DNA samples.

2. Materials and Methods

2.1. Blood sampling

In the study, whole blood samples (n = 5) were collected from bovines after slaughter. Blood samples were taken into vacutainers with EDTA. The blood samples were stored in a deep freeze (-20˚C) until DNA isolation (~3 months). Ethical permission was taken from Van Yüzüncü Yıl University Animal Researchers Local Ethics Committee (Approval 07.03.2019, 2019/2).

2.2. DNA isolation

Frozen blood samples in deep freezer were thawed, and then 200 µl of thawed blood samples were added to microcentrifuge tubes. These tubes were centrifuged at 10,000×*g* for 2 min, 5,000×*g* for 2 min, 1,000×*g* for 2 min, 500×*g* for 2 min, and 100×*g* for 2 min. After centrifugation at defined levels, 100 µl supernatant was taken carefully and added to new microcentrifuge tubes. Thereby, pellet and supernatant were separated. Since starting blood volume should be 200 µl, 100 µl PBS were added onto both pellets and supernatants. In the control group, direct 200 µl FTB samples were added to microcentrifuge tubes, and DNA extraction was carried out directly. DNA extractions were performed by using a spin-column-based DNA extraction kit (GenEx^{TM} Blood, Genall).

2.3. Determination of DNA quality and quantity

DNA concentrations (ng/µl), A260/A280 and A260/A230 ratios were determined spectrophotometrically (Nanodrop 2000c, Thermo Scientific, USA). Isolated DNA samples were stored in a deep freezer (-20 ˚C) for further analyses.

2.4. Agarose gel electrophoresis of isolated DNA samples

Isolated DNA samples were analyzed by agarose gel electrophoresis. For this purpose, 0.7% agarose gel (w/v) was prepared (Ghatak et al., 2013; Fuentes-Pardo & Ruzzante, 2017). For a better gel result, a thin and narrow gel comb was preferred (Lee & Bahaman, 2012; Arslan et al., 2021). 10 µl isolated DNA samples were loaded into wells and run at 55 volts for 240 min. After running, the gel was soaked into ethidium bromide solution (5 µg/ml) for 30 min. Then, the gels were destained with deionized water for 15 min and visualized under ultraviolet light.

2.5. Real-time PCR analysis of DNA integrity

Quantitative analysis of DNA integrity was evaluated by real-time PCR (long-run) as described in previous studies (Evans et al., 2016; Arslan, 2022). In the long-run real-time PCR, final concentrations of each reaction were 2 µM Syto82 (Life Technologies, USA), 1× buffer B1, 200 µM dNTPs, 2 mM $MgCl_2$, 1 U DNA Polymerase (Hot FIREPol® Solis Biodyne), ~50 ng of DNA template and 500 nM primers in a 20 µL reaction volume. Information about the used primers was given in Table 1. Each reaction was performed duplicate and the average Ct value was used for calculations. DNA standards, 6.25 ng, 12.5 ng, 25 ng, 50 ng, were prepared for standard template dilutions and used to determine PCR efficiencies.

Table 1. Primers used in the quantitative DNA integrity analysis

Primers	5' – 3' sequence	PCR product (bp)	Reference
Long	F:GGCAATACCAGTTGAATTTG R:TGTGTTAGTACGTTCCTAGT	3067	(Arslan, 2022)
Short	F:TTTTATACTCTCAGGATTGAGC R:TTGATATTTTCTTCAGGTACACTAT	70	(Arslan, 2022)

F: Forward, R: Reverse

For long amplicons, PCR conditions were set up as 15 min at 95 °C for initial denaturation and 50 cycles for 15 s at 95 °C for denaturation, 15 s at 60 °C for annealing and 4 min at 72 °C for extention. For small amplicons, PCR conditions were set up as 15 min at 95 °C for initial denaturation and 40 cycles for 5 sec at 95 °C for denaturation, 15 s at 60 °C for annealing, and 1 s at 72 °C for extention. Agarose gel electrophoresis and melting curve analysis were carried out to determine amplicon specificities. DNA integrities were determined by using the previously described equation (Evans et al., 2016).

2.6. Real-time PCR analysis of mtDNA/nDNA ratio

In the isolated DNA samples from the pellet parts, mtDNA/nDNA ratios were determined by real-time PCR. For this purpose, the previously described method was applied (Arslan, 2022). Briefly, each reaction contained 10 µl 2xSyber Green Master Mix (Amplifyme, Blirt), 0.3 µM forward and reverse primers, 1 µl DNA sample, 7.8 µl PCR grade water. Information about the used primers for this analysis was given in Table 2. PCR condition was as follows: 3 min at 95 °C for initial denaturation was followed by up to 40 cycles of 5 s at 95 °C for denaturation, 10 s at 60 °C for anneling, 5 s at 72 °C for extention. Each sample was run duplicate. Melting curve analysis was carried out by gradually increasing temperature (1°C/5s) from 50 °C to 95 °C. The 2ˆ(–delta delta CT) method was performed to evaluate mtDNA/nDNA levels in the experimental groups and control group (Livak & Schmittgen,

2001). In calculations, $Ct_{BTF3-ND1}$ was used to determine nDNA levels in the DNA samples whereas $Ct_{ND1-BTF3}$ was used to highlight mtDNA levels.

Table 2. Primers used in the real-time PCR analysis for evaluation of mtDNA/nDNA levels

Primer	Region	5' – 3' sequence	Reference
ND1-F	mtDNA	AGCCATATCAAGCCTAGCCG	(Arslan, 2022)
ND1-R	mtDNA	TTTGAGTTGGAAGCTCAGCC	
BTF3- F	nDNA	CATGTCCTACACAGGCGAAG	(Arslan, 2022)
BTF3-R	nDNA	GAAATTCGGGAGCTTGGCGG	

F: Forward, R: Reverse

2.7. Statistical analysis

Student's t-test (two-way, unpaired) was carried out to compare groups and determine significance levels. Mean and standard deviations were given as data points in figures. R software, the 'stats' package, was used for statistical analyses (R Core Team) (R, 2017).

3. Results

DNA samples were obtained from pellets and supernatants as described. All the results obtained were given in Figure 1. According to the results, centrifugation at 100×g for 2 min failed to collect intact cells and lysed fractions. In this level of centrifugation, about 40% of the genetic material found in the FTB was lost in the supernatant. Centrifugation at 500×g for 2 min was also insufficient to collect intact or lysed nuclear pellet, where ~85% of genetic material in FTB was collected and ~15% of genetic material remained in the supernatant part. Centrifugation at 1,000×g for 2 min was able to collect about 99% of genetic material found in the FTB. DNA quantity isolated from the supernatant in this level was 0.53±0.45 ng/µl. Also, in this group, isolated DNA yield increased compared to the control group ($P<0.05$). The same situation was also found in 5,000×g and 10,000×g groups (Figure 1). This result suggests that initial elimination of hemoglobin and EDTA increases the column's affinity to bind DNA, resulting in high DNA yield. 5,000×g for 2 min and 10,000×g for 2 min were significant centrifuge levels because no detectable DNA was found in the supernatant part. According to the results, 1,000×g for 2 min and over centrifugation levels were successful to obtain genetic material found in the FTB. 5,000×g for 2 min and 10,000×g for 2 min centrifugation levels led genetic material to be collected successfully. Therefore, these centrifugation levels can be used in the light of the present study.

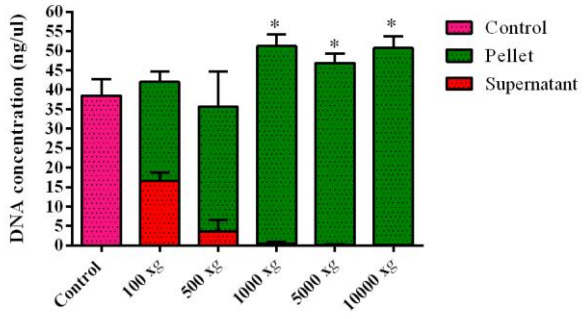

Figure 1. DNA quantity (ng/µl) levels obtained control and experimental groups. * indicates significance level ($P<0.05$) between control and pellet groups.

A260/A230 ratios and A260/A280 ratios in the isolated DNA samples from the control pellet and supernatant were shown in Figure 2. A260/A230 ratios in pellets were not significantly different compared to the control (Figure 2a). A260/A230 ratio decreased significantly in the supernatant part of 500×g for 2 min. A260/A280 ratio in the 10,000 ×g for 2 min group was affected significantly (Figure

2b). However, the value was 1.83±0.01 which was the desired level (Cartozzo et al., 2018). Note that A260/A230 ratios and A260/A280 ratios were excluded in these groups since isolated DNA quantity from supernatants which were obtained by 1,000×g for 2 min and higher centrifugation levels were almost no detectable.

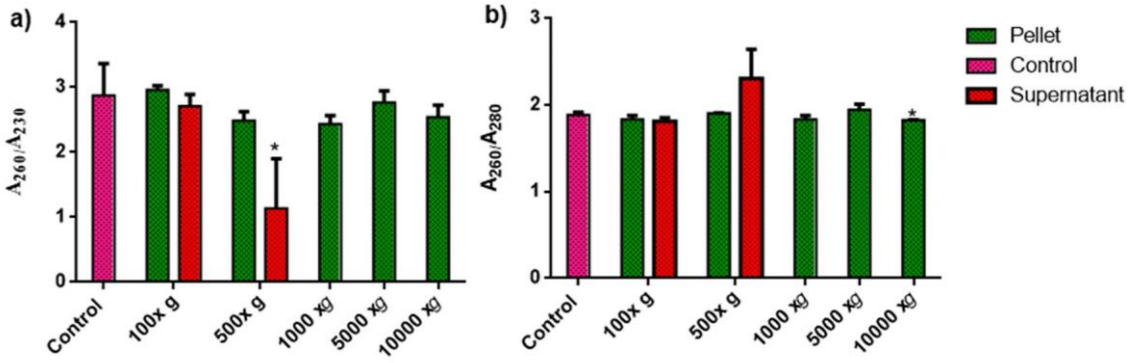

Figure 2. DNA qualities in the control and experimental groups. a) A260/A230 ratios and b) A260/A280 ratios is shown. * indicates significance level (P<0.05) between control and indicated groups.

Isolated DNA samples were also evaluated by agarose gel electrophoresis (Figure 3). According to the results, DNA band intensities decreased in supernatant groups in accordance with spectrophotometric data. 1,000×g for 2 min experimental group gave very low band intensity, where DNA quantity levels were 0.53±0.45 ng/µl, indicating gel sensitivity for checking DNA concentrations. Qualitatively, there was no difference in the DNA integrities in the supernatant parts. Similarly, no detectable integrity was determined in the isolated DNA samples from the pellets.

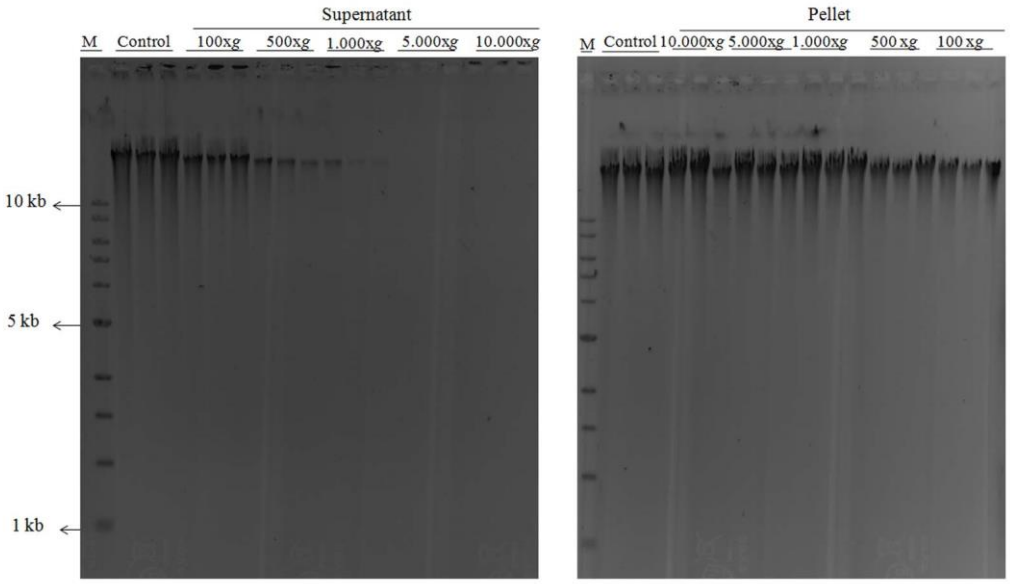

Figure 3. Agarose gel electrophoresis results of the isolated DNA samples from control and experimental groups: supernatants and pellets.

DNA integrity levels were also investigated by real-time PCR using long-run amplification in the isolated DNA from pellet parts (Figure 4). According to the results, in the isolated DNA samples from 100×g and 500×g for 2 min centrifugation levels, DNA integrity levels were similar to the control

groups. However, DNA integrity levels of DNA samples which were isolated from the pellets by 1,000×g for 2 min and over were found to be lower.

Real-time PCR analysis highlighted mtDNA/nDNA ratios in the isolated DNA samples (Figure 5). In this analysis only pellet groups were evaluated. According to the results, relative nDNA levels increased in the isolated DNA samples from pellets obtained by 100×g and 500×g for 2 min centrifuge levels, while in the pellets obtained by centrifugation 1,000×g and over, did not change (Figure 5a). Relative mtDNA levels in the DNA isolated from pellet obtained by 100×g and 500×g for 2 min centrifuge levels decreased significantly, indicating mtDNA loss in the supernatant during these centrifugation levels. However, the ratio did not change in the DNA samples isolated from pellets obtained by centrifugation 1,000×g and over (Figure 5b).

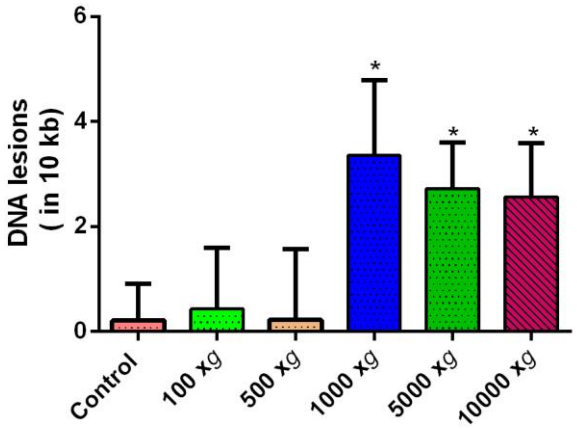

Figure 4. DNA integrity results of the isolated DNA from pellet by defined centrifuge levels.

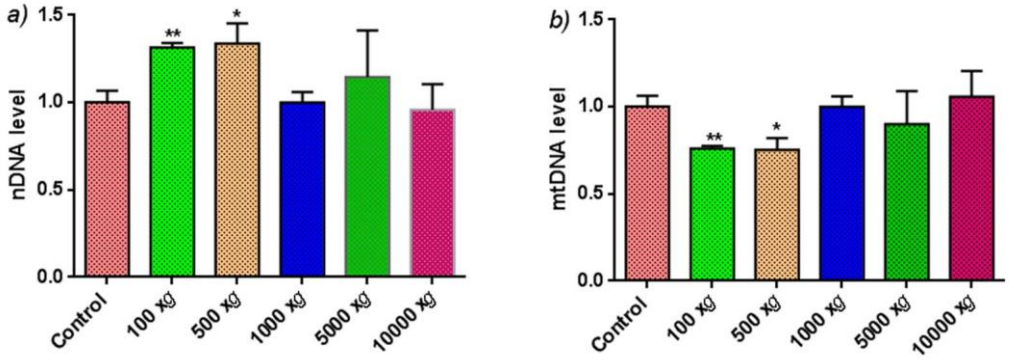

Figure 5. nDNA/mtDNA levels in the isolated DNA samples obtained from control and pellet groups. a) nDNA levels were shown in experimental groups compared to control group. b) mtDNA levels in experimental groups compared to control group. nDNA and mtDNA leves was calculated by using $2^{-\Delta\Delta Ct}$ method based on $Ct_{TERT\text{-}ND1}$ and $Ct_{ND1\text{-}TERT}$, respectively. * and ** indicate significance levels, $P<0.05$ and $P<0.01$, respectively.

4. Discussion and Conclusion

Freezing triggers the lysis of cells due to ice crystal formation (McGann et al., 1988; Steponkus & Lynch, 1989; Cottle et al., 2022). Even though nearly all of the erythrocytes are lysed in the direct freeze-thawing application, most of the leukocytes remain intact (Sloviter, 1962; Lippi, 2012; Arslan, 2022). Recently, it was shown that centrifugation and washing of freeze-thawed blood could be performed before DNA isolation (Arslan, 2022). This application had constructive results on DNA isolation probably due to the elimination of hemolysed fraction including hemoglobin, ETDA, etc. In this approach which centrifugation level is appropriate and their effects on isolated DNA characteristics

are unknown. In the current study, different centrifugation levels changing from 100×g to 10,000×g were used to harvest intact or lysed fraction of FTB, and DNA isolations were carried out from both obtained pellet and supernatant, and isolated DNA characteristics were investigated for the first time.

Centrifugation level at 300×g for 5 min can be used to harvest cells in the whole blood (Dagur & McCoy, 2015). This centrifugation level is higher than 500×g for 2 min. As shown in Figure 1, ~15% of genetic material was found in the supernatant part in this centrifugation level. Therefore, it is not an appropriate centrifugation level. Centrifugation at 1,000×g for 2 min and over can be used for harvesting genetic material found in FTB (Figure 1). In the current study, it was shown that centrifugation force at 1,000×g for 2 min pelleted significantly (~99%) genetic material found in intact or lyzed cells from FTB (Figure 1). This centrifugation force was used to harvest intact cells (Dagur & McCoy, 2015). The result may support that leukocytes are not completely lysed in FTB (Arslan, 2022). This might be due to the subcellular structure of leukocytes, absorbing ice-induced damage. Even though leukocyte lysis occurs limited level, DNA in cells is packaged by histone proteins (Olins & Olins, 1974). This organization increases the molecular weight of cellular DNA to be pelleted by centrifugation. Centrifugation at 5,000×g for 2 min and over pelleted the genetic material found in the FTB successfully (Figure 1 and Figure 3). According to the results, centrifugation force 5,000×g and over levels, DNA isolation from pellet resulted in high-quantity DNA isolation. This result was also highlighted in a recent study (Arslan, 2022). These results strongly implied that decreasing hemoglobin and EDTA before DNA binding increased membrane saturation and binding of DNA to spin-column, leading to increased DNA yield and purity.

In the current study, DNA quality (A260/A230) in the isolated DNA samples from pellets was not affected significantly (Figure 2). However, the value increased in the previous study (Arslan, 2022). This could be due to the different volume or composition of binding and washing buffers used in commercial DNA extraction kits.

Agarose gel electrophoresis was used for DNA integrity checks in the isolated DNA samples. However, no detectable difference was observed (Figure 3). Therefore, quantitative analysis was carried out by long-run real-time PCR. On the other hand, agarose gel electrophoresis showed a detectable DNA band in isolated DNA samples from supernatant obtained by 1,000×g for 2 min. An earlier study reported that agarose gel electrophoresis could be used for DNA quantification, and the detection limit of agarose gel (stained by ethidium bromide) was 5 ng (Tweedie & Stowell, 2005). This result was in accordance with the present study since loaded DNA quantity was ~5.3 ng in isolated DNA samples from supernatant obtained by 1,000×g for 2 min. However, there was no detectable DNA band in the isolated DNA samples from supernatant obtained by 5,000 and 10,000×g for 2 min centrifugation levels. Therefore, these centrifuge levels can be used successfully for DNA isolation from pellet obtained from FTB. Although DNA isolation carried out from pellets obtained by 1,000×g and over resulted in high DNA quantity, DNA integrity level was affected (Figure 4). A similar result was also found in the previous study (Arslan, 2022). Decreasing hemoglobin and EDTA might increase DNA binding to the membrane of the column. This might increase capturing of fragmented DNA by ice-induced DNA breaks (Heard, 1955; Trusal et al., 1984; Lahiri & Schnabel, 1993). DNA integrity can be important for some studies including comparative genomic hybridization (CGH) and whole-genome amplification approach or targeting long amplification size (Wang et al., 2007; Craig et al., 2012; Lucena-Aguilar et al., 2016). When DNA integrity is important, fresh blood samples can be used for extractions or washing of FTB samples should be applied.

mtDNA and nDNA levels were not affected in the isolated DNA samples from pellets obtained 1,000×g or over levels (Figure 5). However, in the previous study, DNA isolation carried out from pellet obtained from FTB at 10,000×g for 1 min was affected significantly (Arslan, 2022). This difference could be due to the use of different binding and washing buffers in the commercial DNA isolations kits.

To conclude, centrifugation at 1,000×g for 2 min and over is convenient for DNA isolations from the pellets obtained from FTB samples. To completely harvest genetic material in the pellet, centrifugation at 5,000×g for 2 min and over is useful. It should be noted that binding and washing buffers used may affect isolated DNA quality and quantity. Pelleting FTB and elimination of hemolyzed fraction by centrifugation and/or washing would enable researchers to obtain more desired DNA from FTB.

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