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Stability of some electrolytes and thyroid hormone concentrations in repeated freeze-thaw serum of Karadi sheep

ABSTRACT

This study aimed to evaluate the effect of repeated freeze-thaw cycles on some electrolytes' concentration (Na⁺, K⁺, Ca⁺ and Cl⁻) and thyroid hormones (T3 and T4), in the serum of Karadi sheep breed. This study was conducted on 18 male and female sheep (aged over one year) in two lines of Karadi sheep Jeshana and Jaff in Sulaimaniyah province. Blood samples were collected from each animal and allowed to clot for 45 to 60 min, then centrifuged at 3000 × g for 10 min to separate the serum. The serum sample was frozen at (-40) degrees and was monitored for 18 months. Serum sample analyzed after (18 months) of storage. Our result showed that repeated freeze-thaw cycling has significant and relevant increases of serum T4 (Thyroxine hormone) in both male and female groups without affecting T3 (Triiodothyronine hormone). Na⁺ and Cl⁻ in both male and female groups showed a significant difference compared to the control group while the remaining electrolytes K⁺ and Ca⁺ didn't show any relevant changes. This study has demonstrated that repeated freeze-thaw cycles do not cause changes in some biochemical constituents studied in sheep serum.

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INTRODUCTION

In the Kurdistan region, sheep breeding makes a major contribution to the agrarian economy, with a population of 3,500,000 heads (Animal Production and Veterinary Directorate, 2011). Repeated freezing and thawing of plasma may influence the stability of plasma (or serum) constituents. Results of analyses performed in plasma samples exposed to repeated freeze-thaw cycles might therefore differ from analyses performed in fresh, or only once thawed samples.

Many previous researches that looked at the effects of different storage temperatures or freeze/thaw cycles used serum samples from animals (Cray et al., 2009; Hu et al., 2020; Reynolds et al., 2006; Thoresen et al., 1992; Thoresen et al., 1995). Although most of the researches done are on human serum or plasma (Männistö et al., 2007; Männistö et al., 2010; Cuhadar et al., 2012; Ismail, 2017; Comstock et al., 2001; Reyna et al., 2001; Nwankpa et al., 2018). In addition, previous research focused on the impact of freeze/thaw cycles on peptide hormones and other blood constituents (Hillebrand et al., 2017; Männistö et al., 2007; Vlot et al. 2018). Furthermore, the temperature at which certain analytes are stored influences their stability (Oddoze et al., 2012; Cray et al., 2009; Boyanton and Blick, 2002; Heins et al., 1995). Electrolytes are compounds when dissolved in water become ions, and can conduct electricity (Houston and Harper, 2008; Bishop et al., 2010). Body fluid volume and osmotic control (Na⁺, K⁺, and Cl⁻), myocardial rhythm and contractility, neuromuscular excitability (K⁺), as well as acid-base balance (K⁺, Cl⁻) are all

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processes in that electrolytes play a role in (e.g., K^+ , Cl^-) (Mcpherson and Pincus, 2011; Bishop et al., 2010).

There are few studies on the effects of freezing, thawing and storing on serum thyroid-stimulating hormone (TSH) and thyroid hormones free thyroxine (fT4) and free triiodothyroxine (fT3). (Oddie et al., 1979; Kashiwai et al., 1991; Koliakos et al., 1999). Thyroid hormone levels, body weight and energy expenditure have long been known to be linked. (Fox et al., 2008; Iwen et al., 2013; Knudsen et al., 2005).

Todini (2007) claims that proper thyroid gland function and thyroid hormone (TH) activity are critical for maintaining productive performance in domestic animals (growth, milk, and hair fiber production), and circulating TH can be considered as indicators of the metabolic and nutritional status of the animals (Riis and Madsen, 1985). Thyroid hormones are involved in nutrient absorption, metabolism, and calorogenesis (Todini et al., 2007), growth and development (Nixon et al., 1988), and reproduction (Blaszczyk et al., 2004).

This study aims to evaluate the effect of repeated freezing and thawing processes on concentrations of electrolytes (Na^+ , K^+ , Ca^+ , and Cl^-) and thyroid hormone concentration (T3, and T4) in the serum of two Karadi sheep lines.

MATERIAL AND METHODS

This experiment was performed at the College of Agricultural Engineering Sciences, University of Sulaimani.

Experimental Animals

Two different genetic lines of Kurdish local sheep were used in the study, Jaff from Halabja and Jeshana from Sulaimani governorate. The experiment was carried out on 18 male and female sheep, aged (over one year) old, with a mean weight of (34.05 ± 2.62 kg).

Collection of blood sample and serum

Blood samples were collected from each animal once and allowed to clot for 45 to 60 min, then centrifuged at $3000 \times g$ for 10 min to separate the serum.

- Serum samples were frozen at (-25) degrees and were monitored for 18 months, as in April 2019 samples were obtained in both locations.
- Serum sample analyzed after (18 months) of storage as we have mentioned below.

Freeze-Thaw cycles and biochemical analysis:

The first test (Jan-19-2021), referred to as T1 (Control group)

After 18 months of storage, the plasma samples were thawed at room temperature for approximately 1 h until completely thawed, and tests were done for all samples, then they were re-frozen at $-25^\circ C$.

The second test (Feb-4-2021), referred to as T2

After 18 months of storage, the plasma samples were thawed at room temperature for approximately 1 h until completely thawed and then was re-frozen at $-25^\circ C$. This process was repeated four times by means: (freeze-thaw Jan-23-2021), (freeze-thaw Jan-27-2021) and (freeze-thaw Jan-31-2021), lastly (freeze-thaw Feb-4-2021).

The third test (Feb-19-2021), Referred to as T3

After 18 months of storage, the plasma samples were thawed at room temperature for approximately 1 h until completely thawed, and then were re-frozen at $-25^\circ C$. This process was repeated nine times by means: (freeze-thaw Jan.- 19-2021), (freeze-thaw Jan-23-2021), (freeze-thaw Jan-27-2021), (freeze-thaw Jan-31- 2021), (freeze-thaw Feb-4-2021), (freeze-thaw Feb-8-2021), (freeze-thaw Feb-12-2021), (freeze-thaw Feb-16-2021), lastly (freeze-thaw Feb-20-2021).

At all three times (1st, 2nd, and 3rd tests), the following biochemical analysis was performed after thawing the plasma of the samples T4 and T3 and determined by using the Electrochemiluminescence immunoassay (ECLIA) method to measure the T3 and T4 concentrations (nmol / L) using Cobas e 411 automated analyzers. A commercial kit was used (Roche Diagnostic GmbH, and Mannheim, Germany). Serum electrolytes concentration (mmol/L): (Na^+ , K^+ , Ca^+ and Cl^-) were determined using Electrolyte by Ion-Selective Electrode (ISE) technique (Convergent - Germany).

Statistics

One-way analyses of variance (ANOVA) with repeated measurements were used to compare differences in component levels after freeze-thaw cycles with baseline denoted as the component level after the first thaw. Data are presented as least squares (mean \pm SD). Significance was declared at $P < 0.05$. We used the Duncan test and also Dunnett test to figure out all changes between the test and also compared to the control group (T1).

RESULTS AND DISCUSSION

T1 is used as a baseline measurement for T2 (Five freeze-thaw cycles) and T3 (Ninth freeze-thaw cycles), as generally stored samples are thawed once (Gislefoss et al., 2017).

Mean concentration values with their standard deviations of T3 (nmol/L), T4 (nmol/L), Na⁺ (mmol/L), K⁺ (mmol/L), Cl⁻ (mmol/L) and Ca⁺ (mmol/L) in serum of Jeshana male and female are shown in table (1).

T3 (Triiodothyronine hormone) in both male and female groups didn't show any significant difference in both tests T2 (five freeze-thaw cycles) and T3 (Nine freeze-thaw cycles) when compared to the control group (T1), T4 (Thyroxine hormone) in both male and female groups showed a significant difference in the third test that is after nine freeze-thaw cycles when compared to the control group and the second test that is after five-freeze-thaw cycles, but there wasn't any significant difference between the control group and the second test that is after five freeze-thaw cycles.

Na⁺ showed a significant difference between the female group at the fifth and ninth freeze-thaw cycles respectively when compared to the control group, while there wasn't any significant difference between the male groups. Cl⁻ showed a significant difference in both male and female groups after the nine freeze-thaw cycles when compared to the control group, while there wasn't any significant difference between the T1 (control) and the T2 (fifth freeze-thaw cycle). The remaining electrolytes K⁺ and Ca⁺ didn't show any significant difference during the whole processes of freezing-thawing cycles as showing in table (1).

Table 1: Effects of repeated freeze-thaw cycles on some serum parameters in Jeshana male and female sheep (mean \pm SD).

Hormone and minerals	Experimental Groups					
	Jeshana Male			Jeshana Female		
	T1	T2	T3	T1	T2	T3
T3 (nmol/L)	3.95 \pm 0.23	4.03 \pm 0.26	3.80 \pm 0.22	4.14 \pm 0.12	4.52 \pm 0.14	4.16 \pm 0.13
T4 (nmol/L)	126 ^b \pm 0.21	131.8 ^b \pm 0.21	206 ^a \pm 0.16	124.4 ^b \pm 0.05	124.4 ^b \pm 0.13	188.2 ^a \pm 0.16
Na ⁺ (mmol/L)	151.25 \pm 0.04	138 \pm 0.09	144.2 \pm 0.03	152.6 ^a \pm 0.03	144 ^b \pm 0.02	144.3 ^b \pm 0.01
K ⁺ (mmol/L)	4.73 \pm 0.03	4.54 \pm 0.11	5.2 \pm 0.03	4.82 \pm 0.07	4.87 \pm 0.08	5.2 \pm 0.09
Cl ⁻ (mmol/L)	91 ^a \pm 0.08	90 ^a \pm 0.12	68.3 ^b \pm 0.1	84.2 ^a \pm 0.06	88.8 ^a \pm 0.04	74.4 ^b \pm 0.01
Ca ⁺ (mmol/L)	0.5 \pm 0.25	0.51 \pm 0.24	0.62 \pm 0.3	0.60 \pm 0.21	0.61 \pm 0.19	0.75 \pm 0.22

* Different letter in the same row within the same group mean significant differences ($P < 0.05$), absent of letters mean no significant difference between treatments.

Mean concentration values with their standard deviations of T3 (nmol/L), T4 (nmol/L), Na⁺ (mmol/L), K⁺ (mmol/L), Cl⁻ (mmol/L) and Ca⁺ (mmol/L) in serum of Jaff male and female (sheep) are shown in table (2).

T3 (Triiodothyronine hormone) in both male and female groups didn't show any significant difference in both tests T2 (five freeze-thaw cycles) and T3 (Nine freeze-thaw cycles) when compared to the control group (T1). T4 (Thyroxine hormone) in both male and female groups showed a significant difference between the second test that is after five freeze-thaw cycles and also

the third test that is after nine freeze-thaw cycles comparing to the control group that is after one freeze-thaw cycle.

Na⁺ in both male and female groups showed a significant difference between T1 (control group) with T2 (five freeze-thaw cycles), and also T3 (nine freeze-thaw cycles). Cl⁻ showed a significant difference in both male and female groups of T2 (five freeze-thaw cycles) and T3 (nine freeze-thaw cycles) comparing to the T1 (control group), but there wasn't any significant difference between T2 and T3. The remaining electrolytes K⁺ and Ca⁺ didn't show any significant difference during the whole processes of freezing-thawing cycles.

Table (2) Effects of repeated freeze-thaw cycles on some serum parameters in Jaff male and female sheep (mean ± SD)

Hormone and minerals	Experimental Groups					
	Jaff male			Jaff female		
	T1	T2	T3	T1	T2	T3
T3 (nmol/L)	1.54 ± 0.24	1.80 ± 0.21	1.45 ± 0.26	1.68 ± 0.12	2.18 ± 0.14	1.80 ± 0.2
T4 (nmol/L)	48.35 ^b ± 0.21	70.8 ^a ± 0.24	87.3 ^a ± 0.33	44.5 ^b ± 0.29	83.18 ^a ± 0.22	102.40 ^a ± 0.3
Na ⁺ (mmol/L)	168.8 ^a ± 0.03	152 ^b ± 0.01	150.8 ^b ± 0.01	165.8 ^a ± 0.03	152.04 ^b ± 0.02	150.8 ^b ± 0.02
K ⁺ (mmol/L)	5.75 ± 0.07	5.62 ± 0.06	6.13 ± 0.04	5.64 ± 0.06	5.6 ± 0.07	5.94 ± 0.07
Cl ⁻ (mmol/L)	58.7 ^b ± 0.09	75.2 ^a ± 0.04	73.8 ^a ± 0.05	70.6 ^b ± 0.07	80.92 ^a ± 0.04	78 ^a ± 0.01
Ca ⁺ (mmol/L)	0.83 ± 0.26	0.81 ± 0.27	0.99 ± 0.19	0.83 ± 0.34	0.9 ± 0.35	1.3 ± 0.28

* Different letter in the same row within the same group mean significant differences (P<0.05), absent of letters mean no significant difference between treatments.

Previous research has shown that storage of serum at room temperature had no impact on some thyroid hormone concentrations. According to Männistö et al. (2007), samples stored for up to 23 years and underwent through thirteen freeze-thaw cycles didn't show any significant changes in their free T3 compared to fresh samples, and thus their findings in line with ours as after nine freeze-thaw cycles and storage for about 18 months T3 hormone didn't show any significant change in all stages of freeze-thaw cycles. Hillebrand et al. (2017) found no effects of multiple freeze-thaw cycles up to four cycles for most of the analyzed endocrine parameters including T3 except plasma renin activity, and this is the same as our results.

Contrary to our findings, the results for Männistö et al. (2010) showed that the concentration of T3 is higher in frozen and thawed samples. As from their results, we conclude that following long-term storage T3 concentrations are quite uniform. Therefore, freezing and thawing itself may result in higher T3 concentrations, as increased during each freeze-thaw cycle, although an increase is seen in our results in the fifth thawing cycle, but doesn't cause any significant difference among them.

According to Hillebrand et al., (2017) freezing and thawing of serum up to eight times did not affect concentrations of any hormone studied among them T4 and according to their work T4 in all types of samples was very resistant to degradation by contact with cellular elements of blood, long-term storage after centrifugation, hemolysis, and repeated freezing and thawing. Serum may be stored for 8 days at room temperature without affecting concentrations of T4, while it showed significant changes in our results for some of the parameters after nine freeze-thaw cycles and some others after five cycles of freezing and thawing.

Various studies examined how storage conditions affected the stability of various serum electrolytes, Gislefoss et al. (2017) stated that sodium was significantly affected by multiple freeze-thaw cycles in serum which is consistent with our results. The results of this finding are contradictory to those found in a recent study by Paltiel et al. (2008) they noticed that sodium was the most stable component both when it came to repeatedly freeze-thaw cycles and long-term storage. Also, Reynolds et al. (2006) indicated that repeated freeze-thaw cycles had no clinically

relevant effect on serum sodium. However, it alone cannot be used as a marker from sample integrity, but primarily as an indicator of volume changes.

In this study, chloride showed a significant difference in both male and female groups, while in most of the published researches chloride is one of the serum electrolytes that showed no significant changes during the whole processes of freezing-thawing cycles (Cray et al., 2009; Reynolds et al., 2006).

Following the current study, Reynolds et al., (2006) found that potassium and calcium in serum were stable and not affected by repeated freezing and thawing. Furthermore, in the results of studies conducted by Cuhadar et al. (2012) and Gislefoss et al. (2017) they conclude that storage time and freeze-thaw cycles did not affect serum calcium and potassium respectively. Our findings were consistent with these results.

Various and contrary effects have been published in the literature regarding the impact of the freeze-thaw cycle on the stability of some electrolytes and thyroid hormone concentrations in frozen serum sheep. If the samples have to be thawed and again refrozen for every measurement, this may negatively influence the results as some hormones are not resistant to freeze-thaw cycles, fortunately, steroid hormones are in general not affected by freeze-thaw cycles. (Vlot et al. 2018; Hillebrand et al., 2017; Männistö et al., 2007).

Storage of samples has been recognized as an important factor in human clinical pathology (Boyanton and Blick, 2002; Clark et al., 2003; O'Keane and Cunningham, 2006). Differences among the cited studies may be due to differences in sample handling before serum separation, storage temperatures, and test methodologies. Similar studies have been limited to canine and avian blood samples, which revealed interspecies differences in storage stability (Hawkins et al., 2006; Reynolds et al., 2006).

We can say that most of the changes between Jeshana and Jaff samples for example in the case of T4 and Na result may be because of pre-analytical conditions, as from a recent study that is done in Sulaimani province diagnostic labs, the analysis showed a high prevalence of improper sample handling during the pre-analytical phase the percentage error was as high as 39% in relevant samples. Hemolyzed samples 9%, erroneous sample identification 8%, and clotted samples were the most common grounds for rejection 6% (Najat 2017).

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استقرار بعض الإلكتروليتات وتركيزات هرمون الغدة الدرقية في دورات التجميد والذوبان المتكررة في مصل الأغنام الكرادي
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الخلاصة

هدفت هذه الدراسة إلى تقييم تأثير دورات التجميد والذوبان المتكررة على تركيز بعض الأملاح (Na^+ ، K^+ ، Ca^{+2} و Cl^-) وهرمونات الغدة الدرقية (T3 و T4) من سيرم سلالة الأغنام الكرادي. أجريت هذه الدراسة على 18 من الضأن من الذكور والإناث (تزيد أعمارهم عن سنة) في نوعين من الضأن الكرادي (الجاف و الجيشانة) في محافظة السليمانية. تم جمع عينات الدم من كل حيوان وتركت تتخثر لمدة 45 إلى 60 دقيقة، ثم طردت (طرد المركزي) عند $3000 \text{ rpm} \times 10$ لمدة 10 دقائق لفصل السيرم. جمدت عينة السيرم عند (-25) درجة وتم رصدها لمدة 18 شهر. تم تحليل عينة السيرم بعد (18 شهر) من التخزين. أظهرت النتائج التي توصلنا إليها أن تكرار دورة التجميد والذوبان المتكرر له زيادات كبيرة وذات صلة في مصل T4 (هرمون التيروكسين) في كل من مجموعات الذكور والإناث دون التأثير على T3 (هرمون ثلاثي يودوثيرونين). أظهر Na^+ و Cl^- في كل من مجموعات الذكور والإناث فرقاً كبيراً مقارنة بالمجموعة كونترول بينما لم تُظهر المتبقية K^+ و Ca^{+2} أي تغييرات ذات صلة. أظهرت هذه الدراسة أن دورات التجميد-الذوبان المتكررة لا تسبب تغييرات في بعض المكونات البيوكيميائية التي تمت دراستها في مصل الأغنام.

الكلمات

المفتاحية:

هرمونات الغدة الدرقية ،
الإلكتروليتات ،
مصل مجمد ،
الاستقرار ،
غنم كردي