RESEARCH

Blood Glucose Analysis with Different Blood Sampling Techniques in Chick Embryos

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Abstract: Chronic Diabetes mellitus (DM) is a metabolic disease with multiple etiologies. It is characterized by hyperglycemia resulting from defects in insulin secretion, action or both. The combined approach of human, in vitro and animal studies is probably the best strategy to improve our understanding of underlying mechanisms. Diabetic animal models are frequently used in the investigation and treatment of genetic and environmental factors in the emergence of DM and complications due to DM. In this study, we aimed to measure blood glucose levels in chick embryos by using different blood sampling methods and to find the most applicable blood sampling method. 50 specific pathogens free (SPF) fertilized chick eggs (White Leghorn chicken) were used in this study. 50 SPF eggs were used for blood sampling on 18th day of incubation. Blood sampling from SPF eggs were made using five different techniques on the 18th day of incubation. 10 SPF fertilized eggs were used for each technique. The stages of chick embryos were determined as stage 44 according to the Hamburger-Hamilton classification. Blood glucose level was measured in only three of the techniques (First, second and fourth technique). The technique with the highest mean blood glucose level (153.90±9.73) was determined as the fourth. The lowest mean was detected in the second technique (130.50±8.20). As a result, it is not possible to say that the three techniques are alternatives to each other. The second technique differs from other techniques according to the Bland-Altman method.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease with multiple etiologies. It is characterized by hyperglycemia resulting from defects in insulin secretion, action or both1. Diagnostic tests are recommended for the diagnosis of DM. These tests include plasma glucose 2 hours post-load after 75 g oral glucose tolerance test (OGTT); glycated hemoglobin (HbA1c) level and a blood glucose value measured randomly in the presence of diabetes signs and symptoms. People with fasting plasma glucose values of ≥ 7.0 mmol/L (126 mg/dl), 2-h post-load plasma glucose ≥11.1 mmol/L (200 mg/dl), HbA1c ≥6.5% (48 mmol/mol); or a random blood glucose ≥11.1 mmol/L (200 mg/dl) in the presence of signs and symptoms are considered to have diabetes[1–3].

The combined approach of human, in vitro and animal studies is probably the best strategy to improve our understanding of underlying mechanisms[4]. Animal models have served a prominent function in the development of the present ideas of pathogenesis and approaches to therapy[5]. Diabetic animal models are frequently used in the investigation and treatment of genetic and environmental factors in the emergence of DM and complications due to DM. There are many studies on experimental diabetes models in the literature[4,6–8].

However, conventional rodent and large animal mammalian models face ethical, practical or technical limitations. Among vertebrates, birds are phylogenically closer to mammals. Among birds and chicks are preferred models in developmental biology, toxicology, cancer research, immunology and drug testing. Chicken eggs can be easily accessible, and they have a short incubation period. Since developing chick embryos are not considered to be animals for up to ten days of embryonic life, its use in experiments does not evoke serious ethical issues[9]. Experimental studies on chick embryo models are often preferred due to their low cost, accessibility, simplicity and reproducibility[10].

Blood sampling from hatchings is less common. But it is essential for issues such as identifying changes in embryonic development. Blood sampling from young chicks requires skill and training. It can also be problematic in terms of possible side effects such as stress and physical injury [11]. In smaller birds, access to blood vessels is more difficult. Blood vessels are usually very small and narrow.

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However, smaller eggs have thinner eggshells[12]. With the difficulty of obtaining blood sampling from chick embryos, it is also important to obtain the desired amount of blood sampling. Enough blood sample is needed to determine values such as blood glucose and insulin levels.

There are many studies in the literature that measure blood glucose levels of chick embryos with different methods. However, it is determined that chick embryos on the same incubation day have very different blood glucose levels[9,13–17]. In this study, we aimed to measure blood glucose levels in chick embryos by using different blood sampling methods and to find the most applicable blood sampling method.

MATERIALS and METHODS

This study was performed in Afyonkarahisar Health Sciences University, Medicine Faculty, Department of Anatomy. Ethics committee approval was obtained from Afyon Kocatepe University Animal Ethics Committee (Date: 12/17/2020, Decision No: 49533702/329).

50 specific pathogens free (SPF) fertilized chick eggs (White Leghorn chicken) were used in this study. SPF eggs were obtained from Izmir Bornova Veterinary Control Institute (Izmir/Turkey). SPF eggs weighed 65±5 g. SPF eggs were placed in the incubator with sharp ends pointing down to ensure continuity of the embryos. These fertilized eggs were accepted as day 0. SPF eggs were incubated at 37.5±0.5 0C and 65-80% humidity in an incubator that performed automatic rotating every 2 hours. 50 SPF eggs were used for blood sampling on 18th day of incubation. Devices and kits that used to measure blood glucose levels are shown in Table 1.

Table 1. Material List

Blood Parameters	Strip	Device
Blood Glucose	Accu Chek Performa Nano Blood Strip (Roche, Basel, Switzerland)	Accu Chek Performa Nano Glucometer (Roche, Basel, Switzerland)

Blood sampling techniques

Blood sampling from SPF eggs were made using five different techniques on the 18th day of incubation. The stages of chick embryos were determined as stage 44 according to the Hamburger-Hamilton classification [18]. All SPF eggs were examined under the light source for determining the air sack. Then the egg shell above the air sack was sterilized with 70% ethyl alcohol. The egg shell was removed with 15 cm curved tip dissection penset. It was aimed to measure blood glucose levels by trying different blood sampling techniques.

First technique

The shell and inner shell membrane of chick embryos were carefully removed with 12 cm extra pointed penset. While removing shell membrane, no damage was done to any vessels. Blood glucose strip was inserted under the thickest blood vessel and the vessel was raised up so that the blood flow continued. Albumen around the vessel was removed with a dry ear stick. No blood sample could be taken from the vessel on the strip with a tuberculin needle. Thereupon, the blood vessel on the strip was dissected with a tuberculin needle. The blood spreading on the strip was aspirated with a tuberculin injector [19]. Blood glucose levels were measured by glucometer (Accu Chek Performa Nano Glucometer Kit, Roche, Basel, Switzerland), (Figure 1).

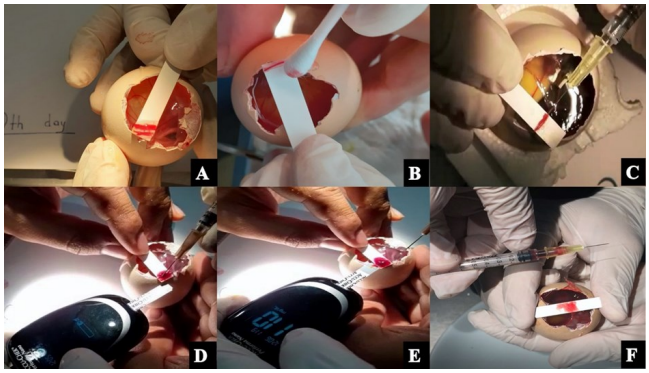

Figure 1. Blood sampling with the first technique. (a) Blood glucose strip was inserted under the thickest blood vessel, (b) Albumen around the vessel was removed with a dry ear stick, (c) Blood sample could not be taken from the vessel, (d) The blood vessel on the strip was dissected with a tuberculin needle, (e) The blood was aspirated with a tuberculin injector, (f) Blood glucose measurement

Second technique

After the eggshell on the air sac was cleaned with %70 ethyl alcohol, the eggshell was carefully opened with 15 cm curved tip dissection penset. The ear stick was moistened with distilled water. The moist ear stick was rubbed gently on the inner shell membrane. The white color of the inner shell membrane which did not show the underlying structures was made transparent. Thus, the blood vessels became visible under the shell membrane. In this way the most suitable vessel (thick and large) for blood sampling was determined. Then, the shell membrane was pulled in the same direction as the vessel with the dry ear stick and inner shell membrane was stretched. Vessel was brought closer to the shell membrane. Blood was collected from this vessel with 30GX13 mm mesotherapy needle attached to the tip of the 1 ml disposable insulin syringe (Figure 2).

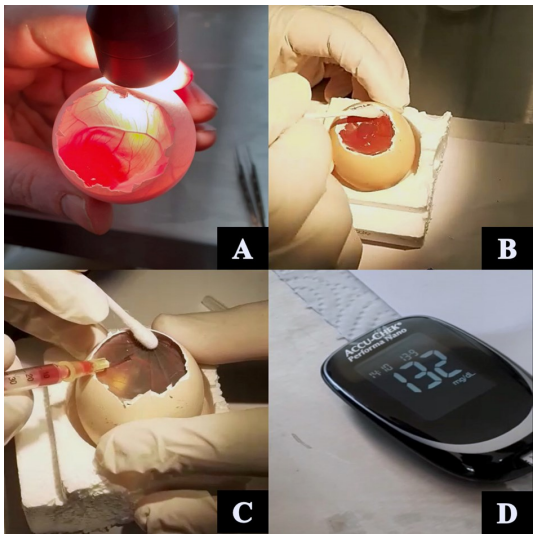

Figure 2. Blood sampling with the second technique. (a) The eggshell was carefully opened, (b) Transparency with a moist ear stick, (c) Taking a blood sample, (d) Blood glucose measurement

Third technique

After removing the egg shell with 15 cm curved tip dissection penset, the inner shell membrane was carefully removed with 12 cm extra pointed penset. Meanwhile, attention was paid to blood vessels. Then, blood sampling was attempted from the thickest vessel with the 30GX13 mm mesotherapy needle attached to the tip of the 1 ml disposable insulin syringe. Blood sampling was not possible with this technique because the blood vessel could not be fixed (Figure 3).

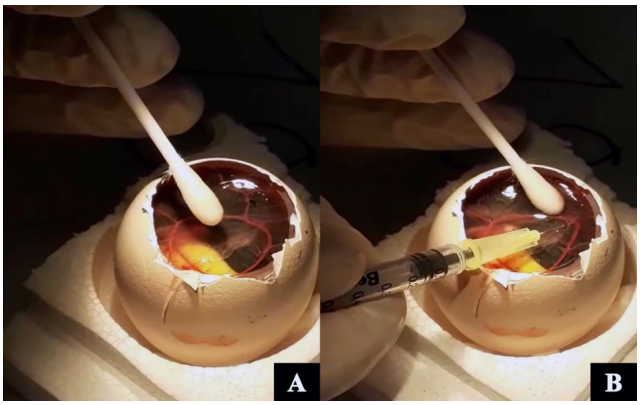

Figure 3. Blood sampling with the third technique. (a) The inner shell membrane was carefully removed, (b) Blood sampling could not be taken from the thickest vessel

Fourth technique

After removing the egg shell with 15 cm curved tip dissection penset, the inner shell membrane was carefully removed with 12 cm extra pointed penset. Then, the embryo was placed on a sterile plate. The amniotic membrane around the embryo was quickly removed with 15 cm curved tip dissection penset. Chick embryo was placed in the supine position, the sternum and ribs were cut with sterile scissors. The heart surrounded by a pericardium was seen in the middle of thoracic cavity. The pericardium was carefully dissected with 12 cm extra pointed penset. Hearts of all chick embryo were beating. Then, 30GX13 mm mesotherapy needle attached to the tip of the 1 ml disposable insulin syringe. An attempt was made to draw blood from the heart. As the amount of intravascular blood decreased due to bleeding, blood sampling was not possible with this technique (Figure 4). Afterwards, the heart was dissected, and the blood glucose level was measured directly from the heart by glucometer (Accu Chek Performa Nano Glucometer Kit, Roche, Basel, Switzerland).

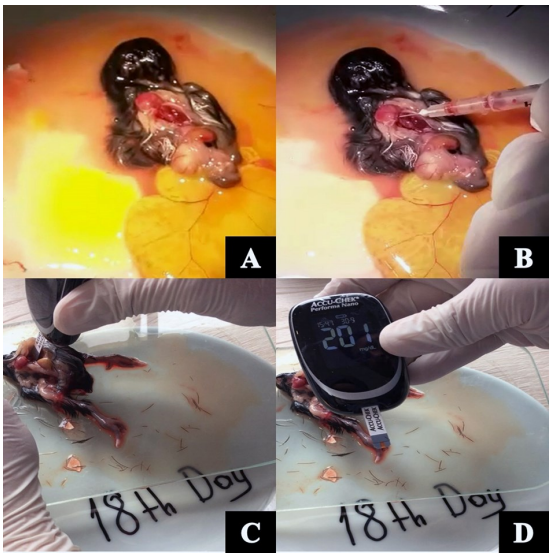

Figure 4. Blood sampling with the fourth technique. (a) Opening the chest wall, (b) Taking a blood sample from the heart, (c) Placing the blood strip in the heart, (d) Blood glucose measurement

Fifth technique

As in the fourth technique, chick embryos were taken into a sterile plate. The vitelline vessels were quickly determined. Blood sampling was attempted from vitelline vessels with 30GX13 mm mesotherapy needle attached to the tip of the 1 ml disposable insulin syringe. Blood sampling could not achieve with this technique (Figure 5).

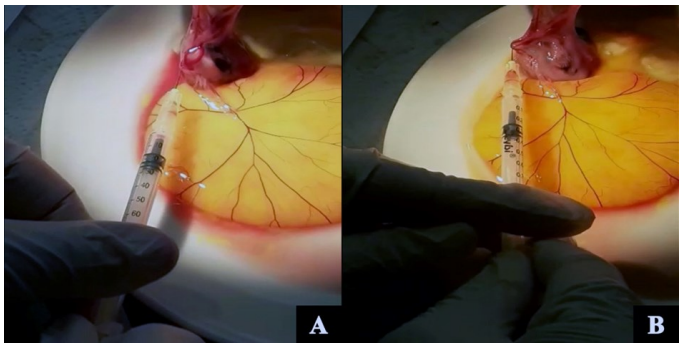

Figure 5. Blood sampling with the fifth technique. (a) Chick embryos were taken into a sterile plate, (b) Blood sampling could not be taken from vitelline vessels

Statistical analysis

Statistical analysis of the data was performed with the IBM Statistical Package for the Social Sciences (SPSS 25.0) program. The Shapiro-Wilk test was used to determine the normal distribution of data. Kruskal-Wallis test was used to compare the blood glucose levels (blood glucose levels of blood sampling taken with three different techniques) since the data were not normally distributed and $n<30$. Dunn test were employed as post-hoc tests and $p<0.05$ were considered significant. Mean statistical values were expressed as Mean±Standard Deviation (Mean±SD). Bland-Altman method was used to determine whether a measurement technique can reliably represent another measurement technique [20,21]. MedCalc statistical software program was used for this method.

RESULTS

In this study, the relationship between five blood sampling techniques and blood glucose levels were investigated. 10 SPF fertilized eggs were used for each technique. Blood glucose level was measured in only three of the techniques (First, second and fourth technique). Mean blood glucose levels according to blood sampling techniques were shown in Table 2.

According to Table 2, the technique with the highest mean blood glucose level (153.90±9.73) was determined as the fourth. The lowest mean was detected in the second technique (130.50±8.20). In the comparison made according to blood sampling techniques, a statistically significant difference was found between first and second techniques and second and fourth techniques ($p<0.05$). However, there was no difference between the first and fourth techniques ($p>0.05$).

Bland-Altman method was applied with the MedCalc software program. The graphs regarding deviations of blood glucose levels from the mean were obtained (Figure 6). When Figures 6a and 6b were examined, it was seen that the differences in blood glucose measurement results obtained according to the two techniques was not systematically distributed and showed a random distribution. According to the results of the analysis in Figures 6a and 6b, it could be said that there was a harmony between the two methods and they were alternatives to each other. Thus, first and second techniques according to Figure 6a; first and fourth techniques according to Figure 6b could be used as alternatives to each other.

Figure 6c showed that the differences of the measurement results showed a systematic distribution around zero and there was a clear relationship between the differences and the means. According to the results of the analysis, it could be said that there was no harmony between the second and fourth methods and cannot be used as alternatives to each other. As a result, it could be stated that the second technique was different from other techniques and could be used in blood glucose measurement.

Table 2. Average blood glucose levels according to blood sampling techniques

	18th Day of Incubation Blood Glucose Level (mg/dL)				
Blood Sampling Technique	n	Mean±SD	Minimum	Maximum	p
First Technique	10	146.10±7.57[a]	136	159	<0.05*
Second Technique	10	130.50±8.20[b]	120	143	<0.05*
Fourth Technique	10	153.90±9.73[c]	138	166	0.585*
Total	30	143.50±12.88	120	166	-

*Kruskal-Wallis test, Dunn test as post-hoc test
a: The difference was determined compared to second technique.
b: The difference was determined compared to fourth technique.
c: The difference was not determined compared to first technique.

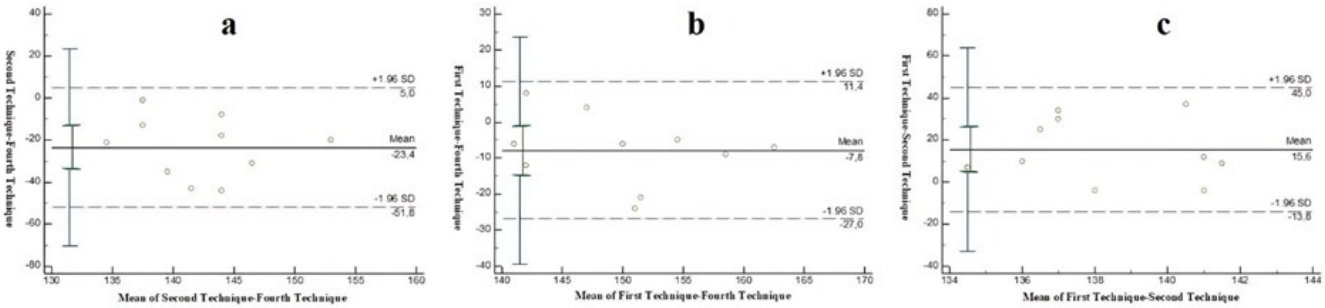

Figure 6. Bland-Altman chart. (a) Comparison of measurement first and second techniques, (b) Comparison of measurement first and fourth techniques, (c) Comparison of measurement second and fourth techniques

DISCUSSION

In this study, blood glucose levels were determined using different blood sampling techniques. The mean blood glucose levels obtained with three blood sampling techniques (first, second and fourth techniques) were shown in Table 2.

Blood glucose concentration is often used as a biological evaluation index in studies evaluating the effects of dietary ingredients on growing conditions and health abnormalities. Studies on blood glucose level determination using many experimental animal models are available in the literature. In studies on blood glucose in avian, measurements were made using whole blood. However, it is difficult to find consistency from blood collection techniques to measurement methods and results[22–25].

Avian have higher blood sugar levels than mammals. Even fasting blood sugar levels are less likely to change[26]. In the literature, there are differences in blood glucose analysis studies performed in chick embryos and newly hatched chickens[23,24]. In addition, there are differences between techniques in terms of mean blood glucose levels. Considering the literature, it was determined that blood glucose levels of blood sampling taken from chick embryos on similar incubation days (18th incubation day) were different from each other[14–17]. In our study, the mean blood glucose level of three techniques was determined as 143.50±12.88 mg/dL.

In the study of Vladimirov et al., blood glucose levels were determined by taking blood sampling from the umbilical artery on the 18th day of incubation of chick embryos. As a result of the study, they stated that the blood glucose levels were between 144 and 150 mg/dL[16]. Thommes et al. collected blood sampling from the umbilical vein of chick embryos on the 18th day of incubation. The mean blood glucose level was 227.9±16.7 mg/dL[14]. Shi et al. reported that blood glucose levels in blood sampling taken from the heart of chick embryos on the 18th day of incubation varied between 150 and 160 mg/dL[17]. In the study of Lu et al., they examined the blood glucose levels of chick embryos from the 16th incubation day to the 13th day after hatching. They found that the blood glucose levels of the 18th day of incubation in the blood sampling taken from the heart of the chick embryo varied between 190 and 215 mg/dL[15]. In our study, differences were found between the mean blood glucose levels of blood samples taken by three different techniques. These results can be explained by the differences in blood sampling techniques. Therefore, Bland-Altman method was used to analyze the harmony between three different blood sampling techniques. The most important feature of this technique is that it objectively reveals the measurement differences between techniques. It can be said that there is no harmony between the second and fourth blood collection techniques examined according to Bland-Altman method.

The advantage of the second technique, we have applied in our study over other techniques is that we first make the inner shell membrane transparent by using distilled water and consequently we can easily see the vessel from which we want to take blood under the membrane. In addition, unlike the third technique, since we do not damage the inner shell membrane mechanically, the vessel from which we want to take blood is not disconnected from the membrane and the vessel can be fixed easily. We think that one of the most important reason for our difficulty in taking blood in the fifth technique, we have applied in our study is the bleeding in the other vessels while taking the chick embryos to the external environment and consequently shrinkage of the vitelline vessels. If it is necessary to measure different blood parameters other than blood glucose levels, the first and second techniques can be used because sufficient blood can be taken. However, with the fourth technique, only enough blood can be taken to measure blood glucose level. In terms of ease of use and applicability, the second technique has been found to be preferable technique than the first technique.

Conclusion

As a result, it is not possible to say that the three techniques are alternatives to each other. It is thought that different blood glucose levels in the literature are due to the difference in blood sampling methods related to which organ or vessel is used and how it is taken. The absence of similar results in the literature can be explained in this way. The second technique differs from other techniques according to

the Bland-Altman method. In this context, we think that the second technique can be used in terms of both statistical and applicability. This result show that this technique can be used as an alternative approach in future studies.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; EA, EB, AB and AV conducted the experiments, EA, AB, and AE made the data analysis and wrote the manuscript.

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