Original Article

In vitro Study of the Effect of High Temperature on Erythrocytes in Sickle Cell Trait

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Abstract

Background: Although, sickle cell trait (SCT) was considered a benign condition with most patients being asymptomatic, however, there is an impressive increase in the number of sudden deaths in the military recruits and athletes, which has led to SCT as a rapidly emerging medical issue. Genetic factors have been extensively investigated in the etiopathogenesis of SCT but, environmental factors have not been studied in depth. **Aims:** The main aim of this study was to investigate the effect of high temperature on the red blood cells (RBCs) in those with SCT and compare this to a control group lacking SCT. **Patients and Methods:** Heat stress to RBCs was induced by *in vitro* incubation of freshly drawn blood at high temperatures (45°C for 35 min). Additional information such as hemoglobin (Hb) level, RBC count, mean corpuscular volume (MCV), and hemoglobin S level was obtained from the medical record of the case and control groups. Data were entered in Statistical Package for Social Sciences version 22.0 (IBM Corp, Armonk, NY, United States) and analyzed to examine the research hypothesis. **Results:** A total of 17 blood samples from SCT (HbAS subjects) labeled as cases and 16 samples from controls (HbAA subjects) were included in this study. The results of this study showed no significant change in sickled erythrocytes in SCT in response to *in vitro* heat stress. **Conclusion:** This study's findings appear to suggest that hyperthermia could be excluded as one of the major factors inducing sickling complications during exhausting exercise. Long-term studies in the future are recommended in this area, particularly to assess the effect of high temperature and sudden death in SCT.

Keywords: *In vitro sickling, heat stress, hyperthermia, sickle cell trait*

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Introduction

Sickle cell anemia is an autosomal recessive disorder characterized by a variety of pathological conditions resulting from the inheritance of the sickle hemoglobin (HbS) gene either homozygously or heterozygously.[1] It is estimated that about 300 million people have sickle cell trait (SCT) throughout the world, including nearly 9% of African Americans in the United States (approximately 3 million individuals). In 2010, the estimated worldwide number of neonates affected by hemoglobin S included 5,476,000 (interquartile ratio 5,291,000–5,679,000) having HbAS (SCT) and 312,000 (294,000–330,000) having HbSS (sickle cell disease). Sickle cell disease (SCD) has heterogeneities and regional spatial distribution. Most of sub-Saharan Africa, the Middle East, and India have high allele frequencies in addition to gene flow comparable to the migration flows of Western Europe and the eastern coast of America.[2]

Approximately 4.2% of the population in Saudi Arabia carries the sickle cell gene, and 0.26% of people have SCD. Significant variation exists in the prevalence of SCD in different parts of the country, with the highest

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prevalence seen in the eastern province, followed by the southwestern provinces. The prevalence of SCT ranges from 2% to 27%, and in some areas of Saudi Arabia, SCD prevalence can reach up to 2.6%. Approximately 17% of the eastern province's population carries the sickle cell gene and 1.2% has SCD; this is the highest prevalence in Saudi Arabia.[3] On the other hand, no case of SCD has been diagnosed in certain areas of the central (i.e., cities of Al-Russ, Al-Unaiza, Al-Mesnab, and Bkaria) or the northern provinces (i.e., cities of Qurayat and Al-Jouf). In the southern provinces, both sickle cell homozygotes and heterozygotes are prevalent in all regions except for Farasan Island.[4]

The SCD results from the homozygous transmission of HbS and is characterized by the polymerization of deoxy-HbS molecules causing chronic hemolytic anemia and vaso-occlusive (i.e., vascular occlusion) crisis (VOC) which are the most common reasons for patient's emergency admissions. Vaso-occlusion and hemolysis are generally incompatible with strenuous exercise and lead to sickle cell crisis.[5] SCT, on the other hand, occurs when an individual inherits a normal hemoglobin gene and an abnormal mutated β-globin sickle gene, constituting the heterozygous state (heterozygosity for HBB Glu6Val mutation) for the sickle hemoglobin β-globin gene. SCT is a classic example of a Mendelian single gene disease. It is also a part of the larger group of SCDs, which are a group of genetic disorders characterized by the presence of at least one HbS allele and a second abnormal allele that could allow abnormal hemoglobin polymerization and a symptomatic disorder. Thus, there is significant phenotypic heterogeneity in this group.[6]

At its discovery, SCT was considered a benign condition or a non-disease entity, especially as it had some degree of protective function against falciparum malaria without any painful episodes and no apparent impact on life expectancy.[7] In 1970, the first report indicating an association between SCT and sudden death became available; it noted that there was a significant number of sudden deaths in military recruits with SCT during training at moderate altitudes.[8] This is further augmented by various studies between 1977 and 2010 among 2 million recruits during basic training in the US Armed Forces; black recruits with SCT were at 40 times greater risk of unexplained sudden death than other recruits.[9-12] The autopsy reports from these studies indicated vascular congestion of several body organs by an extensive collection of sickled erythrocytes. The cause of death is usually certified as "complications of sickle cell trait."[13]

Because the complications were uncommon and typically mild, SCT continued to be considered a benign carrier state; however, with an impressive increase in reports, SCT has become a rapidly emerging medical issue.[14,15] In 2010, the US National Collegiate Athletic Association drew attention to the association between SCT and exercise death; a policy and guidance document was developed for athletes with SCT in response to trainee athlete deaths that appeared to be related to SCT.[16]

SCT-related deaths have also been reported in other sports athletes. For example, one study reported that football athletes with SCT had a death rate 37 times higher than that of non-SCT athletes.[17] Deaths from exertional sickling in football players were also reported during pre-season training at a university in New Mexico.[18] Supporting this, a 31-year forensic-based registry study regarding a sudden death in US athletes was reported in 2012. They assembled the first sizeable series of competitive athletes (2,462 individuals), and approximately 23 (0.9% overall; 3.3% African Americans) were found to have SCT.[19] Although the exact pathophysiology and the factors that determine exertional events in SCT are not well understood, there is a strong relationship between cardiovascular collapse and conditions that include heat stress, dehydration, illness, and altitude.[8,10,14,20,21]

During extreme exercise, the body core temperature can increase by 2–3°C and can reach up to 40°C. The body core temperature effectively eliminates large amounts of heat through evaporation; however, the hot climate remains a challenge during training and sports competition as it increases heat-related fatalities.[22,23] In several case reports, heat stress was considered a contributing factor that induced the sudden death of SCT carriers during military training and intense sports activities.[24] Since during exercise a whole host of events (heat, movement, dehydration, etc.) are at work, it is nearly impossible to delineate the individual contribution to these "complications of sickle cell trait."

Therefore, we propose an *in vitro* study in which we intend to evaluate the effect of high temperatures on red blood cells (RBCs) in those with SCT and compare this to a control group lacking SCT. Specifically, we hypothesized that there will be:

"No changes in the percentage of sickled erythrocytes in SCT blood in response to *in vitro* heat stress at 45°C when compared to the percentage of sickled erythrocytes in non-SCT blood."

Materials and Methods

Heat stress to RBCs was induced by *in vitro* incubation of freshly drawn blood at high temperatures (45°C for 35 min). The hanging drop preparation and peripheral

blood films were made from the incubated blood stained with Wright Giemsa stain and examined using the light microscope under oil immersion to quantify the sickled RBCs. Additional information such as hemoglobin (Hb) level, RBC count, mean corpuscular volume (MCV), and HbS level was obtained from the medical record of the case and control groups. The complete blood count (CBC) of one case was not available in the records and the person did not oblige our call for a second CBC.

Data were entered in Statistical Package for Social Sciences version 22.0 (IBM Corp, Armonk, NY, United States) and analyzed to examine the research hypothesis. The statistical analysis included frequency and percentage distributions for qualitative variables and mean and standard deviation for quantitative variables. For the next phase of analysis, we used paired and independent sample "t" tests and Pearson's correlation analyses. However, a preliminary examination of the necessary statistical assumptions showed that the study variables significantly deviated from the assumption of normality. Therefore, both Kolmogorov–Smirnov and Shapiro–Wilk tests confirmed such deviations. This violation of normality led us to use corresponding non-parametric counterparts, which included the Wilcoxon signed-rank test, Mann–Whitney U test, and Spearman's rank correlation tests. The present study did not involve patients' personal information or have any implications for the management plan. Hence, no ethical approval was sought, according to the principles of the Helsinki Declaration.

Table 1: Demographic background of the study participants (Values are means and standard deviations)

	Case (*n*=17)	Control (*n*=16)
Gender		
Male	9 (52.9)	10 (62.5)
Female	7 (41.2)	6 (37.5)
Unknown	1 (5.9)	0 (0.0)
Type of Sampling		
Family screening	6 (35.3)	0 (0.0)
Medical service	1 (5.9)	1 (6.3)
Orthopedic service	1 (5.9)	0 (0.0)
Pediatric screening	1 (5.9)	0 (0.0)
Pre-marital screening	7 (41.2)	15 (93.8)
Unknown	1 (5.9)	0 (0.0)
Age	25.59 (16.32)	32 (18.73)

Results

A total of 17 blood samples from SCT (HbAS subjects) labeled as cases and 16 samples from controls (HbAA subjects) were included in this study. Table 1 presents the background characteristics of these subjects by their demographic information. For both the case and control groups, the number of male participants was higher than the number of females. In the control group, almost all the samples came from pre-marital screening while most participants in the case group were of the two types: pre-marital screening and family screening. The average age of participants in the control group (32.06 ± 18.73 years) was higher than that of the case group (Mean = 25.59 ± 16.32 years).

In the analysis of specified research hypotheses, the first attempt was made to compare between pre-incubation % of sickled erythrocytes and post-incubation RBC in the case and control groups separately. As mentioned in the statistical methodology section, both pre- and post-measurements in each group showed significant variation from normality, the non-parametric Wilcoxon signed-rank test for paired data was used to carry out this comparison. Table 2 shows the descriptive statistics of these pre- and post-measurements for each group along with the test results. The comparison was only carried out for case participants as no changes in pre to post values were observed for any of the control participants. The Wilcoxon signed-rank test result in Table 2 failed to demonstrate any significant difference in pre and post measures, pre-incubation: Median = 0.5, post-incubation: Median = 0.5, z = -0.99, P = 0.32. This result showed no significant change in sickled erythrocytes in SCT in response to heat stress.

Next, the correlation between MCV and hemoglobin S (HbS) level in the SCT group was examined. For the same reason of violating the normality assumption, Spearman's rank correlation instead of Pearson's correlation was employed for this purpose. Computed Spearman's correlation revealed a strong positive correlation between MCV and HbS, r_s (16) = 0.93, P <.0005. The case and control groups of participants

Table 2: Descriptive statistics of pre- and post-incubation % of sickled erythrocytes measures and test results of their comparison

Observations	Control				Case			
	n	Min	Max	Median	*n*	Min	Max	Median
Pre-incubation % of sickled erythrocytes	16	0.00	0.00	0.00	17	0.00	0.25	0.05
Post-incubation % of sickled RBC	16	0.00	0.00	0.00	17	0.00	0.25	0.05
Wilcoxon signed-rank test of pre-post difference			-				*z*=-0.99, *P*=0.32	

were then compared from the aspects of the result of the thermal sensitivity test. The results presented in Table 3 show that all the participants in both case and control groups obtained negative results in the thermal sensitivity test.

In addition to examining the main research hypothesis and questions presented above, the control and case participants were further compared through a few more measurements such as Hb level, RBCs count, MCV, and HbS level. The non-parametric Mann–Whitney U tests were used to conduct these comparisons. Tables 4 and 5 represent the descriptive statistics of these measures in both the case and control. From Table 4, it was observed that there was no statistically significant difference in Hb level and RBCs count of participants in case and control groups. However, the average MCV in control group (Median = 87.20) was significantly higher than that (Median = 74.75) of the case participants, U = 26.50, z = -3.83, $P < 0.0005$. In addition, the average HbS, hemoglobin S level in control group (Median = 00.00) was significantly lower than that (Median = 34.30) in case participants, U = 0.00, z = -5.20, $P < 0.0005$. Table 5 depicts the comparison of case and control group participants with respect to the mean Hb levels, RBC count, MCV, and HbS by Mann–Whitney U test results.

Table 3: Result of thermal sensitivity test by case and control participants

Thermal Sensitivity Test Result	Positive	Negative	Total
Control	0	16	16
Case	0	17	17
Total	0	33	33

Discussion

Most military recruits and athletes with SCT (HbAS) regularly participate in exhausting exercises without realizing that they may be at particular risk. The SCT condition is not a barrier to involvement in military activities, exercise, or participating in sports; however, individuals with SCT should be made aware of their condition as a preliminary step before engaging in exhausting exercise; as a result, they can initiate preventive measures to prevent SCT complications and manipulate the risk factors that may induce exercise-related complications under the SCT condition.

Patients with SCD (HbSS) suffer from tissue damage and life-threatening complications that are primarily caused by the polymerization of HbS and the resulting erythrocyte sickling. The increase in sickled erythrocytes can be correlated with the clinical severity of vaso-occlusive events.[25] In SCT (HbAS), the role of erythrocyte sickling in exercise-related deaths has been suggested from several autopsy reports on military recruits and athletes, whose deaths were considered a complication of SCT. These reports documented vascular congestion of several body organs due to an extensive collection of sickled erythrocytes. Theoretically, intravascular sickling with extensive microvascular obstruction may represent the main pathogenesis explaining SCT complications during strenuous exercise activity.[13,19,20]

Table 4: Descriptive statistics of hemoglobin (Hb), red blood cells (RBCs) count, mean corpuscular volume (MCV), and hemoglobin S (HbS) level of participants in case and control groups

Parameters	Control				Case			
	n	Min	Max	Median	*n*	Min	Max	Median
Hb level	16	83.00	173.00	145.50	16	77.00	172.00	122.50
RBCs count	16	3.64×10^{12}	5.60×10^{12}	4.93×10^{12}	16	3.98×10^{12}	6.84×10^{12}	5.17×10^{12}
MCV	16	69.20	95.90	87.20	16	57.50	86.10	74.75
HbS,	16	0.00	0.00	0.00	17	25.10	41.10	34.30

Table 5: Mann–Whitney *U* test results for comparing case and control group participants with respect to hemoglobin (Hb), red blood cells (RBCs) count, mean corpuscular volume (MCV), and hemoglobin S (HbS) level

Parameters	Case or Control	*n*	Mean Rank	Mann–Whitney *U* Test Result
Hb level	Control	16	18.53	U=95.50, z=-1.23, P=0.22
	Case	16	14.47	
RBCs count	Control	16	13.47	U=79.50, z=-1.83, P=0.07
	Case	16	19.53	
MCV	Control	16	22.84	U=26.50, z=-3.83, P<0.0005
	Case	16	10.16	
HbS, Hemoglobin S level	Control	16	8.50	U=0.00, z=-5.20, P<0.0005
	Case	17	25.00	

In several reports, exercise-induced sudden death in SCT is the ultimate concrescence of an association between extensive exercise, hyperthermia, dehydration, and hyperosmolality, acidosis, and red cell dehydration.[26-29] This leads to extensive erythrocyte sickling and microvascular obstruction. So far, there have been no studies conducted where the effects of high temperature alone on sickling processes were investigated.

The objective of this *in vitro* study was to investigate the effect of high temperature on sickled erythrocyte formation. We hypothesized that there would be no significant change in sickled erythrocytes in SCT in response to heat stress. To test our hypothesis, we designed this *in vitro* experiment to incubate blood samples from SCT at a high temperature. The temperature used in this investigation was greater than the highest reported body temperature during exhausting exercise (45°C); by this method, we estimated the changes in sickled erythrocytes.

The extensive analysis of data by using appropriate, non-parametric statistical methods showed no significant change in sickled erythrocytes in SCT in response to heat stress alone. Thermal stress did not appear to have any adverse effects on erythrocyte sickling. These findings appear to suggest that hyperthermia could be excluded as one of the major factors inducing sickling complications during exhausting exercise. The results of the thermal sensitivity test indicate that the erythrocytes from SCT are not sensitive to thermal stress.

In addition to the results related to the primary hypothesis, a strong positive correlation was found between MCV and HbS and the level of sickle erythrocytes in SCT (HbAS); the maximum detection was 0.25%, which shows the morphology of incomplete/partially sickled erythrocytes. These were characterized by morphological features of sickle-like erythrocytes, which have been described by Wilson and colleagues as hybrid morphology of normal erythrocytes and fully sickled ones.[30] Our findings are novel and unexpected since many prior investigations have implied heat stress as a contributing factor to complications of SCT (exertional collapse and sudden death).[10]

Limitations of the study

The study has a few limitations; the first limitation was the number of sample size, it is important to have a sufficient sample size to conclude a valid research result. The second was the lack of previous research studies on the topic. The third limitation was the lack of other *in vitro* hypoxic stressors such as solubility sickling test to induce the sickling, and then to compare if additional heat stressing will cause a difference in the outcome of our findings. Another major limitation was the nature of this study, which was an *in vitro* study, and the authors were unable to extrapolate their findings to in-vivo situations, such as athletic events. Therefore, we recommend that further study be carried out in patients with SCT following a passive heat exposure (e.g., sauna). This would allow us to delineate the sole contribution of heat to RBC morphology in SCT versus non-SCT.

Conclusion

The results showed no significant change in sickled erythrocytes in SCT in response to *in vitro* heat stress. Negative thermal sensitivity test results were found for both the study subjects. A strong positive correlation between MCV and HbS among SCT subjects was revealed. Long-term studies in the future are recommended in this area, particularly to assess the effect of high temperature and sudden death in SCT. Further research could include prospective studies of a large cohort of subjects measuring a full range of environmental factors, and pathophysiological experiments to characterize the effects of hyperthermia, exercise, and other climatic changes.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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