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Single Time Point Hematological, Biochemical And Cytokines Assays Of Sheepsuffering From Cutaneous Squamous Cell Carcinoma.

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ABSTRACT

The aim of study was to assay some immuneand hematological parameters of sheep cutaneous squamous cell carcinoma (cSCC).Seventy seven sheep suffering from cSSC and five apparently healthy skins were used. They were classified according to the type of cells infiltrating tumor tissues into polymorph infiltrating cSCC and lymphocytic infiltrating cSCC.Blood and tissue lymphocyte activity using glucose consumption test were measured.Some hematological, biochemical and cytokines profile were taken. Multiple sites for cSCCwere obtained with high prevalence in females and aged animals.Comparing with the control healthy sheep, it was found that serum albumin concentration,RBCs count, HCT % and hemoglobinwere significantly decreased ($P<0.05$) in both cSCC groups. There is a significant elevated in neutrophil and lymphocyte count ($P<0.05$) of polymorph infiltrating and lymphocyte infiltrating groups respectively than healthy control group. Lymphocyte infiltrating cSCCinfected sheep showed significantly higher serum level of TNF-α, IL-6 and glucose consumption ($P<0.05$) by peripheral and tissue lymphocytes in comparison with the sheep with polymorph infiltrating cSCC and healthy control ones. However, IL-4 serum level was similar in both types of cSCCs and healthy control sheep. Polymorph infiltrating cSCC in sheep demonstrated a significantly higher serum level of IL-10 and IL-17 ($P<0.05$) comparing to healthy control sheep. We concluded that some parameters associated with lymphocytic infiltrating cSCC can be considered as a better predict for outcome after surgery.In the next study, we have to study tracked profiles over multiple time points in animals and used this data to improve tumor prognosis and prediction.

Keywords: Squamous cell carcinoma, sheep, cytokines glucose consumption test, lymphocyte infiltration.

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INTRODUCTION

Cutaneous squamous cell carcinoma (cSCC) is a commonlyinvasive cancer with low metastatic propertyassociated with cellularabnormalities of epidermis and UV-damaged keratinocytes[1]. cSCC is not only hazardous for humans but also for different domestic species [2]. In Saudi Arabia, cattle, horse, camel, sheep and goats all affected with cSCC[3-4]. cSCC has been reported in different body areas of animals including ocular, ear, lip, back, groins, udder and perineal regions[5].cSCCin different breeds of sheep (Najdi and Naemi) has been recordedespecially in oral part[6].

Over the past decades, especially in arid areas, increased incidence of cSCC to near endemic status has led to high economic loss [7]. The exact causes of cSCC remains unidentified but carcinogenic factors have been well documented including solar radiation, ultraviolet,genetic abnormalities, some chemicals and immunosuppressive agents[7-8]. Surgical removal was the firstinterventionprocedure for cSCC but the complete retrieval was depending on many factors such as sizeand siteof the tumor [9]. Surgery was not the ultimate key to cSCCand does not give the promise of non-returns after removal. Despite the advance in diagnosis and surgical removal in the recent decade, the consequence remains limited by metastasis warning[10].

There is strong signal that immune response plays achiefpart in the development or elimination of different hard tumors [11]. In addition, the effect of inflammatory response might have an influence on the outcome of the tumor[12]. It has also become seeming that both innate and adaptive immune response can endorse tumor advancement as well as facilitate tumor elimination [13].There was a need for understanding the exact role/roles of the immune responsein the tumor sequences. It has been known that cancer progression is not only evaluated by the entire properties of the cancer but also by the activity of animal immune system.

Valuation of tumor infiltrating polymorph cells and lymphocytes in histopathological sections can offer important prognostic evidence in different types of solid tumor, and could also be of value in predicting reply to therapeutics.Tumor infiltrating lymphocytes, for a long time ago was considered as optimal signs for response to therapy [14-15]. However, implementation as a routine cytokines measurement has not yet been accomplished. Scientists have long recognized the immune cells infiltration to the tumor environment, which frequently offer significantdata regarding tumor activities, prognosis and response to different types of immunotherapy[16].Standardization of polymorph infiltrating cSCC and lymphocytic infiltrating cSCC measurements will aid to improve advancement in this arena, augment both the quality and quantity of analogoussigns, and help to thoroughly measure the utility of infiltration in this era of immunotherapeutic approach[17].

Recently, high number of different types of skin multiple mass in sheep has been described in Qassimreagion, KSA. Although the communication between the host immune system and tumor cells is still not fully understood, the present study aimed to examine immune parameters of peripheral and infiltrated polymorph cells and lymphocytes types to be used as optimal biomarkers for response to surgery.

MATERIALS AND METHODS

Animals and grouping: Seventy seven sheep of about 1-6 years old suffering from cSSC were used in the present study. These sheep were admitted from different zones in Qassim region to the Veterinary Teaching Hospital, College of Agriculture and Veterinary Medicine, Qassim University, KSA,with abnormal outgrowths on the skin of different body regions from August, 2016 to July, 2018.Qassim's weather (latitude 20–26 ◦C N, longitude 044–48 ◦C E, altitude of 600-750 m above sea level) is sunny most of the year**[18]**. Annual range of minimum temperature is 5–20 ◦C and that of maximum temperature is 24–46 ◦C. Annual air humidity range is 20–60%. Sheep with skin lesions were exposed to exhaustive inspection, determining breed, age and sex.Natures, gross and microscopic appearance of the solid lesion were subjectedbefore surgical treatment. Five apparently healthy skin 2-3 years of age selected from the Qassim University Farm were used as healthy controls. Specimens were taken from different parts of the surgically excised tissue of skin tumor, fixed in buffered formalin (10%) before embedding in paraffin. Sectionswere stained by using the combination of

hematoxylin and eosin (H&E) [19]. Stained sections were examined under light microscope and pictured using digital camera.

For each lesion,the length (L), the width (W), and the height (H) were calculatedfor tumor volume calculation using special caliber (Manostat)**[20]**. To offer the accurate measurement of tumor frame, the volume of each tumor was calibrated as 0.5 × L × W × H **[20]**. For irregular shape of tumor, each lobe were measured separately and summed to obtain the volume of the whole mass. Dimensions were handled in the anterior-posterior, cranoi-caudal, and transverse directions.According to the distribution of polymorphs and lymphocytes in the tumor tissue by histopathological clarification, cSCCs were categorized into 2 main types; polymorph infiltrating cSCC (n=33) and lymphocyte infiltrating cSCC (n=44). Five apparently healthy skin were used as control(Table 1).

Table 1: Variable distribution of cSCCs in sheep

Variables		Polymorph infiltrating cSCCs (n=33)	Lymphocyte infiltrating cSCCs (n=44)
Breed	Najdi	22	34
	Naemi	11	12
Age	1-2 years	9	13
	5-6 years	24	31
Sex	Male	6	11
	Female	27	33
Volume (cm^3)	Less than 20	6	14
	20-70	9	12
	More than 70	18	18
Site	Tail	7	9
	Flank	12	14
	Abdomen	8	10
	Udder	6	11

Blood samples collection: Ten ml blood samples were collected by sterile jugular venipuncture from control and before surgical operation of tumor infected animals. Each blood sample was divided into two parts;the first part was collected into tubes containing EDTA (Becton Dickinson, NJ, USA) to be used for determination of hematological parameters.Thesecond part was used for separation of serum.

Hematology: A complete blood count (CBC) was carried out using an automated analyzer (VetScan HM5, Abaxis, CA, USA). The hemogramincludesred blood cell count 10^6/µL (RBC), hemoglobin concentration g/dL(Hb), and hematocrit% (HCT) in addition to blood indices of mean corpuscular volume fL(MCV), mean corpuscular hemoglobin pg(MCH) and mean corpuscular hemoglobin concentration g/dL(MCHC).Measurements related to leucogram include total leukocyte count 10^3/µL (WBC), lymphocyte 10^3/µL (LYM), monocyte 10^3/µL (MON), neutrophil 10^3/µL (NEU) and eosinophil10^3/µL (EOS) counts. The serum parameters were measured via an automated biochemical analyzer (VetScan VS2, Abaxis, CA, USA.) to evaluate the protein profile, which included total protein g/dL(TP), albumin g/dL(ALB) and globulin g/dL(GLOB) in addition to creatinineg/dL(CREA), amylase U/L (AMY), blood urea nitrogen g/dL(BUN) and alkaline phosphatase U/L (ALP). Electrolyte profile included calcium g/dL(Ca), phosphorus g/dL(PHOS), sodium mmol/L (Na) and potassium mmol/L (K).

The second part was collected in plain tubes and allowed for one hour after collection to clot for separation of serum by centrifugation at 3000 × g for 20 min. Sera were collected in another clean tubes and kept in a deep freezer at -20 ºC to be used in measurement of cytokines.

Th1-polarized cytokine (TNF-α, IL-6), Th2-polarized cytokine (IL-4, IL-10) and IL-17 measurement: Cytokine concentrations for sheep TNF-α, IL-6, IL-4, IL-10 and IL-17 were determined using sandwich enzyme-linked immunosorbent assay kits (ELISA) purchased from CUSABIO BIOTECH CO., LTD. and following the manufacturer's instructions. The colors were measured spectrophotometrically at a wavelength of 450 nm.

Isolation of peripheral and tumor infiltrating lymphocytes: Lymphocytes infiltrating cSCCwereisolated as described previously **[21]**. Briefly, fresh part of cSCCobtained from excised tissue after surgeryincluding stroma

and epidermis were homogenized after mixing with equal volume ofculture media (RPMI) supplemented with 10%fetal calf serum (FCS). Thehomogenates were placed on a Ficoll-hypaque gradient for discontinuous density gradient separation. Dead cells, RBC, acidophil, basophil, neutrophil and majority of the tumor cells were precipitated to the bottom and the cells at the interface consisting of macrophages, and lymphocytes. The pelleted cells at the gradient interface were washed with culture media. For removal of macrophages,carbonyl iron was added and incubated at 37 c for 30 minutes and then centrifuged for 15 minutes at 2500 g. The supernatantfree macrophage portion was collected and centrifuged at 2500 g for 10 minutes and suspended in 10% FCS. Cell viability wasdone by diluted trypan blue stain(0.4% trypan blue/PBS).Cell number was calculated by hemocytometer.For peripheral lymphocytes, 200µl of respective whole blood was mixed with 2 ml of .1% sodium carbonate solution, incubated for five minutes followed by centrifugation.The precipitate was washed with PBS followed with the RPMI- 1640 culture medium.

Glucose consumption test: It was donetoassess lymphocyte transformationaccording to **[22]**. Phytohaemagglutinin-P (PHA, Sigma-Aldrich) was used as T cell mitogen. Blood and tissue lymphocytes were cultured in triplicate in 24-well culture plates. Each well enclosed 200 µl of culture suspension containing 2×10^6 cells with the addition of 5 µg/mL PHA. The plates were kept in CO2 incubator ($CO^2$5%) and37◦C for three days. Incubation media were separated forglucose estimationby using glucose assay kits at 500 nm. The lymphocyte activity was calculated as the quantity of glucose (mg/dL) consumed minus the glucose concentration of cell culture media of control samples.

Statistical analysis: Descriptive values of data were exposed to one way analysis of variance (ANOVA) using program SPSS for Windows version 19. All values were presented as Mean ± SE. Significant differences were determined by Duncan,s New Multiple Rangeand used to distinct between significant means at $P<0.05$ and$P<0.01$.

RESULTS

Variable distribution of cSCC in sheep including types, breed, age, sex, volume and site are illustrated in table (1).According to histopathologicalclarification, cSCCwascharacterized into two different types; polymorph infiltrating and lymphocyte infiltrating cSCC.Thirty three (42.86%) sheep and forty four (57.14%) had affected with polymorph infiltrating and lymphocyte infiltrating cSCC respectively.Najdi breed was mostly affected with polymorph infiltrating (66.6%)and withlymphocyte infiltrating (77.2%) than Naemi (33.4%)with polymorph infiltrating and (32.8%)withlymphocyte infiltrating.A total of 60 of affected animals (77.9%) were females while males represented 17 animals (22.1%) with nearly equal distribution of both types of cSCC. cSCC insheepwas diagnosed in animals from 1-6 years with high incidence (n=55/71.4%) at old ages (5-6 years) than 21/28.6%at young age (1-2 years). The prevalent sites of cSCC affected are flank (n=26/77);abdomen (n=17/77); udder (n=17/77) and tail (n=16/77). The volume of tumor (cm^3) observed was ranged from small(n=16/77) to medium (n=21/77) to large (n=36/77).

Macro andmicroscopic appearance: The two types of cSCC studied weresimilarly appeared grosslyas erosive horn-like,oval to round structureand sometimes irregular at the outersurface of the skin of flank,abdomen,udderand tailregions (Fig. 1).These structures were ranged in size and sometimes were ulcerated and tinged with bloodas a result of removal of the horn structure by friction. The consistency wasfirm to rigid. Exudation and notable odor were also observed.Histopathplogicalfigures showedarrayof poorly to well differentiated intrusive epithelium associated with inflammation processand infrequently keratin pearls, necrosis, hemorrhage, numerous mitotic records and apoptotic bulks with an enlarged nuclear to cytoplasmic relation were observed in some sections. In this study, according to infiltrating cells, we classify lesions in animals into two groups, polymorph infiltrating cSCC and lymphocyte infiltrating cSCC(Figs. 2&3).

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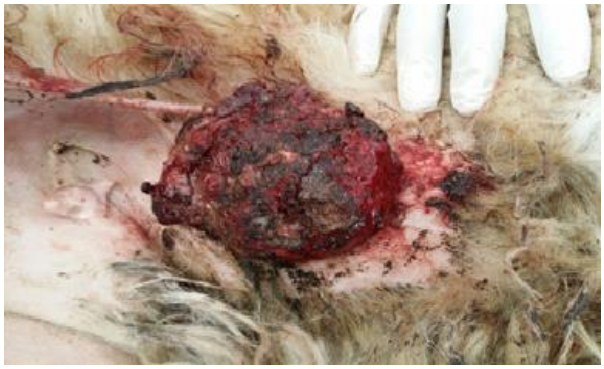

Figure 1: Cutaneous squamous cell carcinoma of abdominal site of sheep.

Biochemical profile: The effect of two types of cSCCs on the protein profile of selected sheep was shown in table (2). Comparing with the control healthy sheep, it was found that albumin concentration of polymorph infiltrating and lymphocyte infiltrating cSCCsgroups was significantly decreased ($P<0.05$). In addition, there was no significant difference between total protein and glucose of two groups of infected sheep comparing to the healthy group. Moreover, globulin concentration was significantly increased in lymphocyte infiltrating SCC group ($P<0.05$) than that of healthy group.

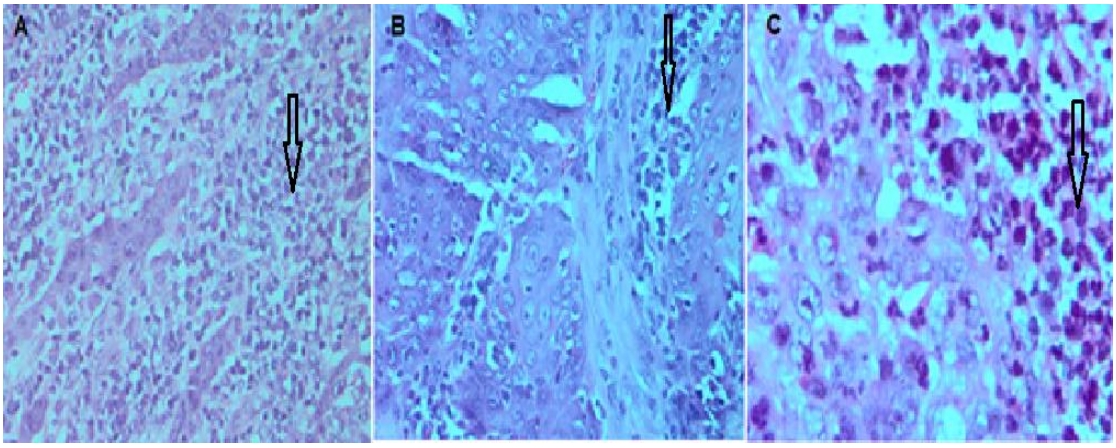

Figure 2: Cutaneoussquamous cell carcinoma in the skin of polymorph cells group presentinggroups of neoplastic cells creating numerous cell nests of various sizes with blood cells and tumor cells with double nucleoli. Some cell nest exhibited epithelial pearls as keratinized centers with the infiltration of polymorph cells (H&E X 10, A, 40, B and 100, C).

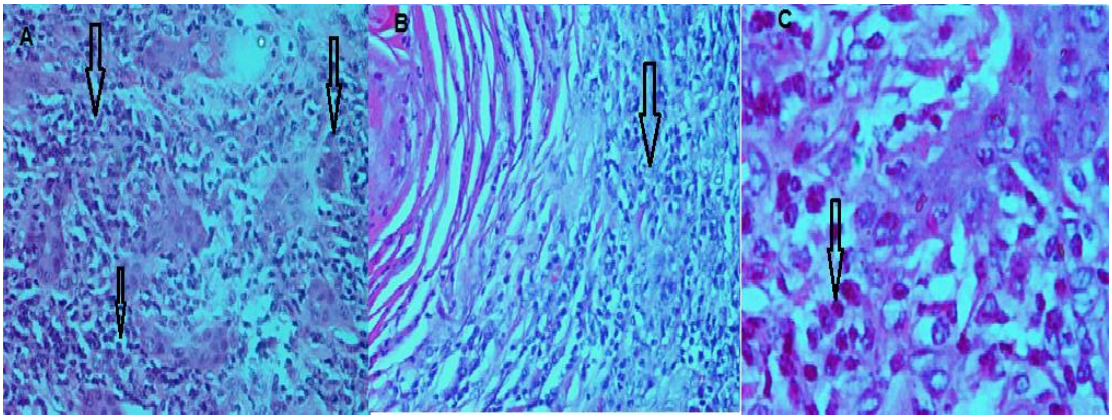

Figure 3: Squamous cell carcinoma in the skin of lymphocytes infiltrating group presenting masses of neoplastic cells constituting numerous cell nests of various size. A dense population of lymphocytes (arrow) is interposed between keratinized centers (H&E X 10, A, 40, B and 100, C).

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Table 2: Effect of two types of cSCC on sheep serum biochemical profile

Groups	Healthy sheep	Polymorph infiltrating cSCC	Lymphocyte infiltrating cSCC
ALB (g/dL)	3.77±0.04	2.33±0.0.2 [a]	2.12±0.03 [a]
GLOB (g/dL)	4.54±0.54	5.11±0.82	5.88±0.03[a]
TP (g/dL)	7.72±1.20	7.44±1.98	7.81±1.33
ALP (U/L)	258.5±12.8	233.5±10.54	254.5±12.73
ALT (U/L)	31.8±3.76	34.65±4.65	33.45±6.85
CREA (g/dL)	0.65±0.32	0.65±0.22	0.64±0.21
BUN (g/dL)	33.67±2.87	35.65±3.09	36.43±3.87
GLU (g/dL)	45.76±6.82	49.76±5.87	50.44±6.01

Data are represented as the mean ± standard error. Letter [a] indicate significance at *P* <0.05, compared with control healthy sheep.

The effect of two types of cSCCson liver and kidney function of selected sheep was shown in table (2). Data revealed that there was no significant difference in CREA and BUN levels of two types of cSCCscomparing to control healthy group. Likewise, ALP and ALT did not show any significant difference in cSCC groups comparing to healthy control group.

Table (3) showed the effect of two types of sheep cSCCs on the leukogram. The results showed that WBCs count of sheep suffered from types of cSCCs is significantly increased (P<0.05) comparing to the healthy control group. However, in lymphocyte infiltrating cSCC sheep, there is a significant increase in LYM count (P<0.05) than control animals. There is a significant elevate (P<0.05) in NEU count of polymorph infiltrating SCC group than healthy control and lymphocyte infiltrating cSCC groups. On the other hand, there is no significant difference in EOS count in two types of sheep cSCCscomparing with control healthy sheep. In addition, MON count did not show any significant difference between sheep in both control and affected groups.

Table 3: Effect of SCC on sheep hematological profile

Groups	Healthy sheep	Polymorph infiltrating SCC	Lymphocyte infiltrating SCC
WBCs (×103/µL)	10.18±2.45	15.65±2.54 [a]	15.54±2.08 [a]
LYM (×103/µL)	65.11±2.88	69.77±2.98	74.11±2.65 [a]
MON (×103/µL)	8.26±1.87	7.32±1.75	7.43±1.32
NEU (×103/µL)	21.54±3.76	34.99±1.43 [a]	26.54±4.52
EOS (×103/µL)	1.03±0.63	1.45±0.88	1.38±0.65
Rbcs (×106/µL)	11.54±3.21	6.34±1.02 [a]	6.04[a]±0.54
HGB (g/dL)	15.73±1.73	10.44 ±1.76 [a]	11.54±1.09 [a]
HCT (%)	41.43±2.44	32.11±3.87 [a]	31.45±3.00 [a]
MCV (fL)	28.53±2.06	21.56 [a] ±1.11	20.56±2.43 [a]
MCH (pg)	13.99±1.66	14.32±2.87	14.31±2.86
MCHC (g/dL)	59.43±5.87	56.11±4.76	55.35±5.81

Data are represented as the mean ± standard error. Letter [a] indicate significance at *P* <0.05 compared with control sheep.

The effect of SCC on the haemogram of selected sheep was shown in table (3). Data revealed that there is a significant decrease (P<0.05) in RBCs count of sheep with both types of cSCCs comparing to that of the healthy control group. HGBconcentration of sheep with two types of cSCCs was significantly lower (P<0.05) than that of the healthy control group. Similarly, the HCT % and MCV of sheep with cSCC was significantly lower (P<0.05) than that of the healthy control group. Moreover, sheep with cSCC did not show any significant difference in MCH and MCHC comparing to control group.

Electrolytes profile: Table (4) showed the effect of two types of cSCCs on the electrolytes profile of selected sheep. Data revealed that there was no significant difference in CAconcentration of sheep neither in healthy control nor in diseased groups. Similarly, PHOS levels did not show significant difference in cSCC compared to

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the healthy control group. In addition, Na and K did not show any significant difference comparing to control groups.

Table 4: Effect of cSCC on sheep electrolyte profile

Parameters	Healthy sheep	Polymorph infiltrating SCC	Lymphocyte infiltrating SCC
CA (g/dL)	10.31±4.21	9.12±1.55	9.43±1.22
PHOS (g/dL)	5.66±2.39	7.41±2.11	6.16±1.43
NA (mmol/L)	151±32	154±12	144±11
K (mmol/L)	4.11±1.65	4.08±1.01	4.87±0.87

Data are represented as the mean ± standard error.

Th1/Th2/IL-17-type cytokine profile in in hydatid naturally infected-animals: The serum level of Th1 pro-inflammatory cytokines, TNF-α, IL-6 and Th2 anti-inflammatory cytokine IL-4, IL-10 andIL-17 in all the sheep, was determined by sandwich ELISA and the results obtained are presented in Table (5). The data revealed that infected sheep with lymphocyte infiltrating cSCC showed significantly a higher serum level of TNF-α and IL-6 in comparison with thesheep with polymorph infiltrating cSCC and healthy control ones. However, IL-4 serum level was similar in both types of cSCCs and healthy control sheep. Polymorph infiltrating SCC in sheep demonstrated a significantly ($P<0.05$) higher serum level of Th-2 anti -inflammatory cytokine; IL-10 and IL-17 comparing to healthy control sheep. However, Th2-anti-inflammatory cytokines serum level was not significantly affected in sheep with lymphocyte infiltrating cSCC compared to healthy control sheep (Table 5).

Table 5: Effect of cSCC on sheep Serum levels of Th1-polarized cytokines (TNF-α, IL-6), Th2- polarized cytokines (IL-4, IL-10,) and IL-17 (ng/ml)

Parameters	Healthy sheep	Polymorph infiltrating SCC	Lymphocyte infiltrating SCC
TNF-α	10.454±2.43	12.109 ±1.22	15.325 ±1.12 [a]
IL-6	9.654±2.76	10.343 ±1.11	16.254±1.76 [a]
IL-4	10.222 ±1.22	11.254 ±2.87	10.497 ±1.76
IL-10	9.661±1.11	15.543±1.10 [a]	9.053 ±1.44
IL-17	10.432 ±1.10	14.109 ±1.22 [a]	10.432 ±2.21

All data expressed as Mean ± SE. Letter [a]within the same row indicate significance at $P<0.05$ compared with control sheep.

Measurements of T-lymphocyte activity was indicated by glucose consumption of pretreated peripheral and tissue lymphocyte with PHA and the results obtained are presented in Table (6). The data revealed that infected sheep with lymphocyte infiltrating cSCC showed significantly higher glucose consumption by peripheral lymphocyte in comparison with the sheep with polymorph infiltrating cSCC and healthy control ones. However,lymphocyte infiltrating cSCC in sheep demonstrated a significantly ($P<0.05$) higher glucose consumption comparing to polymorph infiltrating cSCC infected sheep.

Table 6: Effect of cSCC on glucose consumed (mg\dl) by PHA stimulated tissue and blood lymphocytes of sheep.

Groups	Peripheral lymphocytes			Tissue lymphocytes		
	Glucose in the culture medium (mg/dl)					
	Without PHA	With PHA	Glucose consumption	Without PHA	With PHA	Glucose consumption
Healthy sheep	32.12 ±2.11	23.13 ±3.98	8.22[a] ±3.01	-	-	-
Polymorph infiltrating SCC	34.65 ±3.87	27.76 ±4.09	7.12[b].±2.43	36.30 ±3.54	28.15 ±3.20	7.64[a] ±1.49
Lymphocyte infiltrating SCC	35.48 ±3.65	21.23 ±4.60	14.43[ab] ±3.11	37.63 ±3.24	23.18 ±3.83	14.39[a] ±2.44

Values in the same column with similar letters are significantly different at p< 0.05

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DISCUSSION

Peripheral and tissue lymphocytes constitute the sole effector mechanism of tumor fate and prognosis. However, their impact on cutaneous squamous cell carcinoma (cSCC) remains conflicting. Enduring link between the cancerous cells and the immune or hematological cells might lead to conditions of removal, equilibrium or progression[23]. Microenvironment of solid tumors is not only comprised of malignant cells,but also contains variety of cells. Gajewskiet al. [24]&Coulie et al.[25]showed that the foreign antigens and epitopes of products of tumor cells were considered as antigenic and could induced cell mediated immune response.

The classic measurement of the leukocyte infiltration into tumor tissues that having both pro- and antitumor properties is now reflected the development of cancer [26]. Recent study ofIseki et al.[27]showed that the evaluation of cell infiltration using H-E-stained paraffin sections was easy and might be used to predict the tumor stage and development.Study of Zitvogel et al.[28]showed that the presence of high number of lymphocyte infiltrated the tumor tissue was a good sign of outcome and indicated the participation of immune system in the exclusion of tumor lesion. However, this sign was required the right residence at the right time of lymphocytes [29].Different studies have proved that increased lymphocytes infiltration in tumors associate with tumor grade, step, and tolerant survival [30].Infiltrating lymphocytes could modulate tumorcells by means of attenuating the metastaticpossibility and prognosis[31].Lymphocytesinfiltration was considered as optimal biomarkers for response to therapy [14-15]. On the other hand, decreased lymphocytes in tumor tissue were generally believed to be associated with immune depression, and mightweaken lymphocyte-mediated antitumor immunity[16]. However, it is clear that the presence of tumor infiltrating polymorph cellsdenotes the facilitation ofeither tumor development or regression.Recent worksrecorded that neutrophil infiltrated tumor secreted growth factors that control process of vascular proliferation, cytokines and protease to enhance tumor growth with the end result of poor prognosis[32-33]. Moreover, neutrophils also produced reactive oxygen species, arginase and nitric oxide that suppressed the activity of cytotoxic lymphocytes, natural killer cells, and triggered T cells [34]. Therefore, the present study aimed to find the relationship between peripheral and tissue lymphocytes and polymorphs with some immune and hematological parameters.

In the present work, many cSCCslocated at one or multiple sites as previously described **[2]**.The existence of cSCCwas higher in females and increased with age as previously recorded**[35].** Macroscopically, lesions were erosive horn-like, oval to round structure and sometimes irregular at the outer surface as recorded **[9]**. Ulceration was existed as a result of removal of the cutaneous horn by friction. Histopathplogical figures showed array of poorly to well differentiated intrusive epithelium associated with inflammation process and infrequently keratin pearls, necrosis, hemorrhage, many mitotic records and apoptotic bulks with an increased nuclear to cytoplasmic proportion were noticed in some sectionsas obtained by [5&36]. In the present study, according to infiltrating cells, lesions were classified into two groups, polymorph infiltrating cSCC and lymphocyte infiltrating cSCC.Standardization of polymorph infiltrating cSCC and lymphocytic infiltrating cSCC measurements will aid to improve advancement in this arena, augment both the quality and quantity of comparable signs, and help to thoroughly assess the efficacy of infiltration in this era of immunotherapeutic approach [17].

Comparing with the control healthy sheep, it was found that serum albumin concentration of twotumor groups was significantly decreased. Moreover, globulin concentration was significantly higher in lymphocyte infiltrating cSCC group than that of healthy group. Electrolytes profile, total protein and glucose and liver and kidney function parameters did not show any significant difference in cSCC groups.We concluded that these biochemical parameters not considered as a biomarkers for prognosis based on the types of cell infiltrates.

Measurement of components of CBC in the present study may be used to improved prediction of prognosis as previously mentioned [37-38].Concerning leukogram, the results showed that WBCs count of sheep suffered from two types of cSCCs is significantly higher comparing to the healthy control group. However, in lymphocyte infiltrating cSCCgroup, there is a significant increase in lymphocyte count than control animals. There is a significant elevate in neutrophil count of polymorph infiltrating cSCC group than healthy control and lymphocyte infiltrating cSCC groups. On the other hand, there is no significant difference in eosinophil and monocyte count in two types of sheep cSCCs comparing with control healthy sheep. Data

revealed that there is a significant decrease in RBCs count, HCT % and HGB of sheep with both types of cSCCs comparing to that of the healthy control group. Moreover, sheep with cSCC did not show any significant difference in MCH and MCHC comparing to control group. Other studies found elevation in neutrophil [39], platelet [40]and monocyte [39], and a decrease in lymphocytes [41]was significantly correlated to advanced cSCC with poor survival outcomes. Neutrophil/ lymphocyte ratio was previously considered areflex of systemic inflammatory reaction and was a freelyoffered prognostic marker in multiple tumor types [42-44]. However, another study not finds this relationship[45].

The tumor micro environmental condition plays a vital role in many processes associated with tumor biology as differentiation, immune emission, and metastasis that reflect a continuous relationship between host and tumor. An essential step in the understanding tumor immunology and the inhabitants of immune cells inside tumors is the assay of cytokines[46]. T helper cells are in a core place with respect to tumor lymphocyte immunity. The type cytokines secreted can be classified into mainly three subgroups with distinct properties and in variable percentage: Th1, Th2 andTh17 which originated from naive T lymphocytes (Th0) [47]. Th1 cells produce TNF-α and IL-6 and are considered as pro-inflammatory cytokines, mediate a killer cell response to combat tumor [17]. Th2 cells produce anti -inflammatory cytokine; IL-10, IL-4 and IL-17 that mediated antibody response and considered immune suppressive [48].The balance between stimulatory and inhibitory cues from Th-cells might apply different functional positions [49]. This balance helps in fighting cancer by stimulating the action of CD8+ T-cells to discharge cytokine as mediators [50].However, when abnormalities occur, the balance is troubled, recognized as 'the shifting of Th1/Th2 balance' [51]. This shifting condition might cause different types of diseases including cancer [52]. The present data revealed that infected sheep with lymphocyte infiltrating cSCC showed significantly higher serum level of TNF-α and IL-6 in comparison with thesheep with polymorph infiltrating cSCC and healthy control ones. However, IL-4 serum level was similar in both types of cSCCs and healthy control sheep. Polymorph infiltrating cSCC in sheep demonstrated a significantly higher serum level of IL-10 and IL-17 comparing to healthy control sheep.Lee et al.[53]reported that a larger number of lymphocyteswere correlated with anindependent good prognosis in solid tumor. Polymorph cells were previously considered as antigen-presenting cells, have the ability to stimulate switch to Th2 and favor tumor cell migration and considered as bad prognostic factor[54].Th17 cells have two antagonist actions, in some cases can converted to Th1 with antitumor effects, while in other cases can switch to regulatory T cells with tumor progression [55].Grivennikov et al. [56]reported thatelevation in pro-inflammatory cytokine IL-6 wasaccompanied byharmful effects in several tumors and inflammatory disorders.Th1 cytokines were related to good prognosis in patients with tumor, whereas Th2 cytokines were associated with tumor growth or metastasis [57].This data concluded that most pro-inflammatory cytokines elevated are associated with lymphocyte infiltrating cSCC. In contrast, anti-inflammatory cytokines usually associated with polymorph infiltrating cSCC. These signals provide a therapeutic strategy based on selective blocking of tumor-stimulating cytokines action to stop the survival and growth of tumors, while augmenting the activity of pro-inflammatory cytokines makes the tumor cells more susceptible to the exclusion by host immune system.

T-lymphocyte activity was measured in the present study by glucose consumption of pretreated peripheral and tissue lymphocytes with PHA. The results revealed that infected sheep with lymphocyte infiltrating cSCC showed significantly higher glucose consumption by peripheral and tissue lymphocytes in comparison with the sheep with polymorph infiltrating cSCC and healthy control ones. Peripheral blood and tissue assessments were essential to guesseffectiveness of tumor prognosis and immunotherapy. One assessment was measurement of peripheral immune cell activity [58]. Peripheral immune activation wasprognostic of recovery following surgery [59].

In conclusion, the evaluation of cell infiltration using stained paraffin sections is easy and might be used to identify lymphocyte infiltration for prediction of the prognosis of tumor before surgery. We concluded that some parameters associated with lymphocytic infiltrating cSCC can be considered as a better predict for outcome after surgery.Measurement of immune reaction associated with the cSCCseemed to be an important parameter predicting the regression. This study is performed on single time point assays at the time of surgery. In the next study, we have to study tracked profiles over several time points in animals and used this data to enhance tumor prognosis and prediction.

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