



USING THE HEMATOPOEITIC STEM CELLS TO TREAT THE IMMUNE DEFICIENCY IN EWES

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ABSTRACT

The study was conducted to evaluate the possibility of using the hematopoietic stem cells to treat the immune deficiency in ewes exposed to aflatoxin as immunotoxic substance. Eighteen ewes were subjected to this study, and divided into 3 groups (negative control, positive control and treatment). The results showed significant differences ($P < 0.05$) between groups, as in positive control group the sensitization rate of lymphocyte transformation assay revealed significant decrement ($P < 0.05$), while in the treatment group this rate is elevated significantly and slightly above the levels in control negative group. Results of delayed type hypersensitivity test by using intradermal injection of phytohaemagglutinin showed significant difference ($P < 0.05$) between the negative control, positive control and treatment groups. Whereas levels of IgA are not affected significantly ($P < 0.05$) in negative control and positive control groups, they increased significantly ($P < 0.05$) in treatment group. The levels IgG in positive control group decreased significantly, whereas the levels increased significantly in treatment group. There are no significant differences in levels of IgM between groups of the ewes. It is concluded that the hematopoietic stem cells can be used to treat immunodeficiency diseases in ewes.

KEYWORDS: Immune deficiency, Aflatoxin, Hematopoietic stem cells, lymphocyte transformation assay & DTH test.

INTRODUCTION

Immunodeficiency is disorder in which part of the body's immune system is missing or does not function properly (Lim & Elenitoba-Johnson, 2004), making the animals much more susceptible to infection than their cohorts, thus severe infections are most often encountered in very young, old or immunologically compromised patients (Jones *et al.*, 2004). The domestic animals exposed to several immunosuppressive substances such as aflatoxins which are considered as potent immune suppressant (Agag, 2004). The stem cells are precursor cells capable of proliferation, self-maintenance, production of a large number of differentiated, functional progeny leading to formation of mature cells and tissues, as well as regenerating the tissue after injury, and a flexibility in the use of these options (Potten & Loeffler, 1990; Boulton & Albon, 2004; Melton & Cowen, 2009). Transplants contain the hematopoietic stem cells (HSC) can self-replicate and survive a lifetime in the recipient and regenerate an entire immune system (Shiruzu *et al.*, 2005)

MATERIALS AND METHODS

Animals and Experimental Design

Eighteen Iraqi local breed ewes apparently healthy with age of 2–4 years, were identified by ear tags. The health of all animals was closely monitored before and during the study. All ewes were subjected to detailed clinical examination according to Jackson and Cockroft (2002) before and during the experiment and to delayed type hypersensitivity (DTH) test, lymphocyte transformation assay and estimation of IgA, IgG and IgM by ELISA technique at zero day, 2, 4 and 6 weeks after the last dose of aflatoxin or PBS.

Grouping of the animals

The ewes will be divided into 3 groups as the following:

Group 1 which considered as negative control group (NC): Consist of 6 ewes which were administered with PBS without any immunosuppression and was not received stem cells, but they subjected to all tests mentioned above at the same times of other groups.

Group 2 which considered as positive control group (PC): Consist of 6 ewes administered orally with 64 µg aflatoxin for 14 consecutive days. All animals in this group were subjected to all the tests mentioned above.

Group 3 which considered as treatment group (TRT): Consist of 6 ewes administered 64 µg aflatoxin for 14 consecutive days and treated with 10^7 - 10^9 hematopoietic stem cells (HSC) / Kg BW intravenously in the day 15. All animals in this group were subjected to all the tests mentioned above.

Production of AF

Aspergillus flavus isolate used to produce the AF was obtained from the mycology laboratory of college of Veterinary Medicine – Mosul University, Iraq and cultured in Sabouraud – dextrose agar and identified according to Quinn *et al.* (2004) and the AF was produced according to Shotwell *et al.* (1966) and estimated by ELISA technique.

Isolation, cultivation and marking of the hematopoietic stem cells

Bone marrow biopsy were taken from the sternum, examine and evaluate according to Harvey (2001) and Weiss & Wradrop (2010). Bone marrow was collected into a plastic petri dish with 5 ml of RPMI medium supplemented with 10% fetal calf serum and 50mg/L streptomycin and 100000 units / L penicillin. The harvested bone marrow was gently pipette to break up cell

clumps and the cell suspension transferred to a sterile conical tube. According to Yablonka – Reuveni & Namerof (1987), the bone marrow were subjected to centrifugation at 2000 rpm for 10 minutes, after centrifugation, the fat and serum layers were discarded and the cell pellets were re-suspended with 3 ml of complete growth medium. The cell suspension was loaded on 5ml of lymphoprep (Ficoll – paque, Mediatech, USA). Centrifugation in a cooling centrifuge for 20 – 25 minutes at 2000 rpm at 8 °C in cooling centrifuge was performed. The mononuclear cells were retrieved from the buffy coat layer by a sterile Pasteur pipette and placed in 5 ml sterile conical tube Washing 2 – 3 times with PBS to remove the Ficoll – paque at 200 rpm for 10 minutes at 8 °C. The cell pellet were suspended in 1 ml of culture medium and considered ready for cell counting and viability. Determination of cell number and viability was performed according to pollard & Walker (1997). CD34 and CD45 were used for immunophenotypic analysis of HSC and they performed according to manufacturer instructions (Blue gene, China).

The suspension of MNCs was cultured in RPMI tissue culture supplemented with 10 % FCS at a final concentration of 1×10^6 cell / ml then Incubated at 37° C, 5% CO₂ and fully humidified atmosphere. The growth was inspected daily with 3 – 5 days changing of 50% of the media which was applied as the culture reach approximately to 80 % confluence, the media were aspirated and the cells were washed with 2 ml PBS 2 times, then 2 ml of 0.25 % trypsin – EDTA were added and the flask incubated for 10 minutes. In order to dislodge the cells, the flask was rocked gently, and then 1 ml of culture medium supplemented with 10 % FCS was added. The cells dissociated to a single cell suspension by using a sterile Pasteur pipette and bulb. Then the cell suspension subjected to centrifugation at 2000 rpm for 10 minutes, the supernatant were aspirated and the cell suspended in 1 ml of RPMI plus 10% FCS. Then after the cells were cultured again in similar condition for 2 weeks and re-feed and inspected until they reach to 80 % monolayer confluence (Pollard and Walker, 1997; Al – Azawi, 2003).

Lymphocyte transformation assay

Lymphocyte transformation assay was performed according to methods of Al-Jewari *et al.* (2007)

Delayed type hypersensitivity (DTH) test

Delayed type hypersensitivity (DTH) test was performed to assess the in vivo cellular immunity according to Jacobs *et al.* (1981) and Fernandez *et al.* (2000) by using PHA. The PHA-M was rehydrated with sterile saline to a concentration of 10 mg per ml then 0.1 ml was injected intradermally in the neck region using a 25 gauge needle. Approximately 10 cm from this site, 0.1 ml of saline solution was injected, serving as control.

Enzyme Linked Immuno Sorbent Assay (ELISA)

ELISA was performed according to the manufacture instructions (USBiological, USA) to evaluate levels of IgA, IgG and IgM.

Statistical Analysis

Data were analyzed by using ANOVA. Means were compared byt-test (Steel & Torrie, 1980).

RESULTS

Lymphocyte Transformation Assay

As shown in figure 1. the sensitization rates of the lymphocytes in blood of ewes belong to NC group was not affected during the experiment, and the means of groups at the four consecutive periods are 0.88 (ranged between 0.61 – 1.24) ; 0.9 (ranged between 0.88 – 0.92); 0.95 (ranged between 0.91 – 0.98) and 0.92 (ranged between 0.72 – 1.26) , respectively.

In PC group, the result was different clearly, wherein the sensitization rate decreased significantly ($P < 0.05$) from 0.92 (ranged between 0.77 – 1.1) at zero day to 0.67 (ranged between 0.65 – 0.7) two weeks after aflatoxicosis, and then enhanced reaching to 0.8 (ranged between 0.7 – 0.88) and 0.81(ranged between 0.69 – 0.89) four and six weeks after exposure to AF, respectively. In TRT group the sensitization rate fluctuated slightly with no significant differences between different periods of the experiment, where the means of 0.96 (ranged between 0.89 – 1.06) in zero day became 0.91 (ranged between 0.78 – 1.14) two weeks after aflatoxicosis, then elevated to 0.99 (ranged between 0.71 – 1.2) and 0.97 (ranged between 0.71 – 1.26) four and six weeks after exposure to AF, respectively.

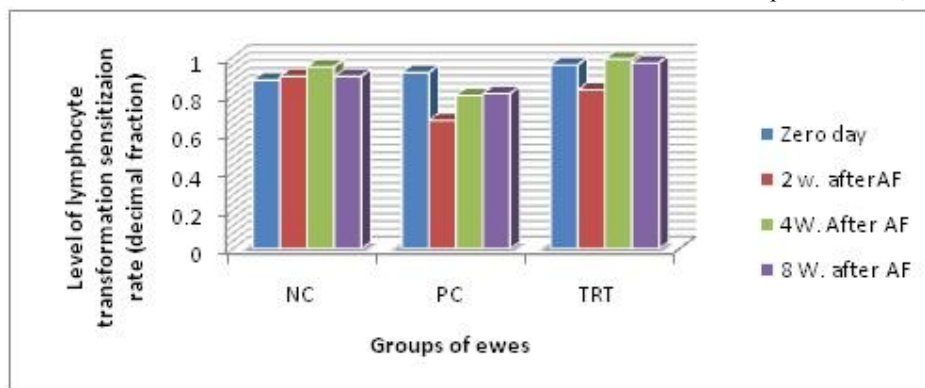


FIGURE 1. Histogram of means of the sensitization rate of lymphocyte transformation assay in all groups.

DTH test

The DTH test also indicated prominent variation between PC group and the remainder groups in response to PHA sensitization, whereas there was no significant differences

($P < 0.05$) in means of the ratio between the increments in skin fold thickness in PHA – injected site before and after injection and saline – injected site before and after injection in NC group as they have 2.59 (ranged between

2.11 – 2.62) ; 2.67 (ranged between 2.34 – 2.83) ; 2.49 (ranged between 2.28 – 2.69) and 2.61 (ranged between 2.29 – 2.72) in the four consecutive periods respectively, whereas in PC group the mean in zero day was 2.12 (ranged between 1.41 – 3.1) which decreased significantly ($P < 0.05$) due to immunotoxic effects of AF to 0.85 (ranged between 0.53 – 1.47) two weeks after exposure to AF and remained decreased but still not significant four weeks after aflatoxicosis where its increased slightly to 1.32 (ranged between 1.16 – 1.49) , but in six weeks after aflatoxicosis the mean retained to 2.1 (ranged between

1.55 – 2.94) to become nearby the mean of zero day . In TRT group , the therapeutic effect of HSC appeared prominently when the means compared with that of PC group , where in TRT group the mean of 2.19 (ranged between 1.43 – 2.8) in zero day remained without significant decrement ($P < 0.05$) at 2.01 (ranged between 1.56 – 2.5) two weeks after aflatoxicosis , while it increased significantly ($P < 0.05$) to 2.9 (ranged between 2.2 – 3.72) and 2.93 (ranged between 2.16 – 3.85) in four and six weeks after aflatoxicosis (figure 2).

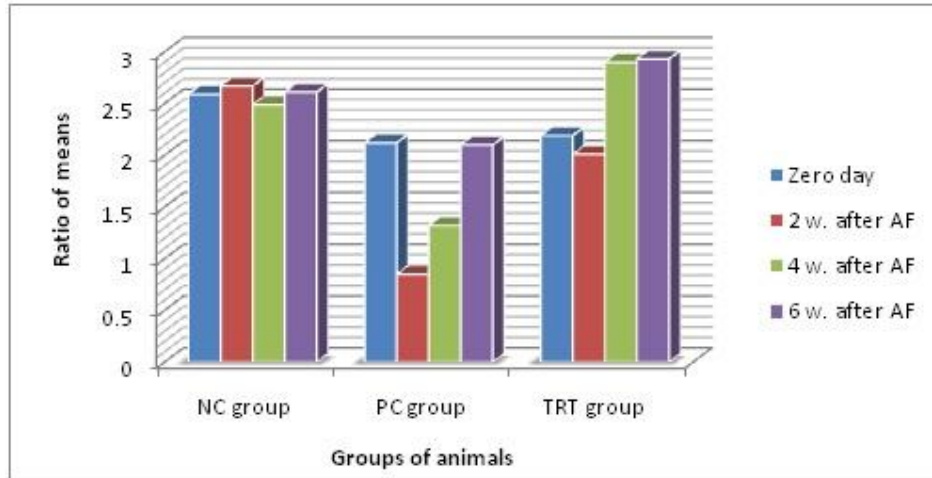


FIGURE 2. Histogram of means of skin thickness ratio test .

Estimation of immunoglobulins by ELISA

a) IgA

The levels of IgA were not affected widely in both NC and PC groups where in NC group the means of this immunoglobulin were 148.75 mg/ml (ranged between 125–193) ; 138.25 mg/ml (ranged between 90 –187) ; 133.88 mg/ml (ranged between 98 – 205) and 126.28 mg/ml (ranged between 86 –155) in the four consecutive periods, respectively, so in the PC the means were 189.02 mg/ml (ranged between 111–231) ; 169.17 mg/ml (ranged between 125 –225) in zero day and two weeks after aflatoxicosis then increased but, not significantly ($P <$

0.05) to 266.13 mg/ml (ranged between 184 – 297) and 264.38 mg/ml (ranged between 202 –285) four and six weeks after aflatoxicosis, respectively. In TRT group , the immunomodulation effect of HSC was appeared markedly in both two and four weeks after exposure to AF , where the means of IgA increased significantly ($P < 0.05$) from 163.19 mg/ml (ranged between 95 –212) in zero day to 978.55 mg/ml (ranged between 794 –1156) and 2123.75 mg/ml (ranged between 1877 – 2426) two and four weeks post exposure to AF , respectively , then after retained to 175.11 mg/ml (ranged between 2.03 – 5.3) six weeks after the exposure.

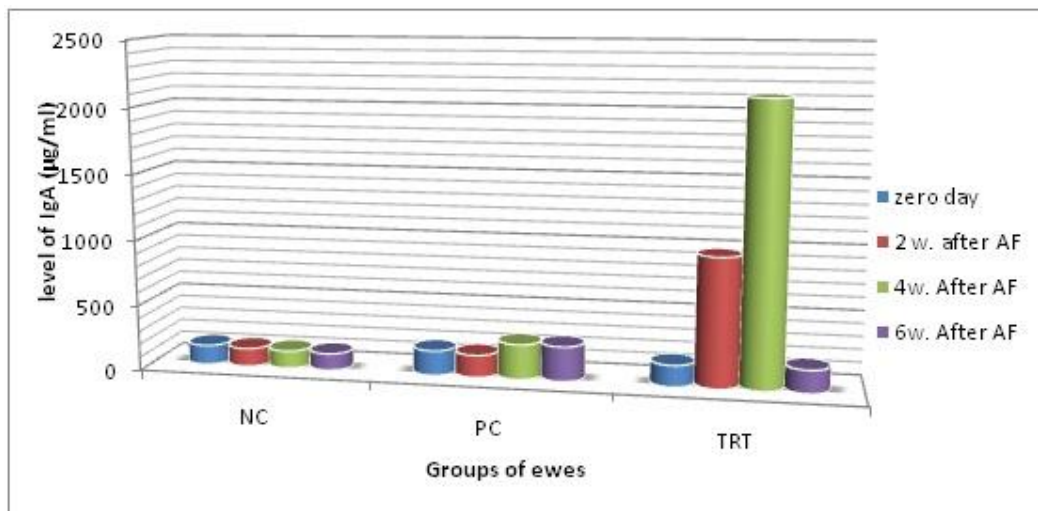


FIGURE 3. Histogram of means of IgA

b) IgG

The means of IgG level in NC were 3.94 mg/ml (ranged between 2.48 –5.3) ; 4.15 mg/ml (ranged between 2.66 – 5.31) ; 4.24 mg/ml (ranged between 3.12 – 4.87) and 4.46 mg/ml (ranged between 3.07 –5.13) in the four consecutive periods. In PC group the means of IgG level increased slightly from 3.34 mg/ml (ranged between 2.03 –5.3) in zero day to 5.96 mg/ml (ranged between 3.87 – 7.21) two weeks after exposure to AF ; but it declined significantly ($P < 0.05$) to 2.17 mg/ml (ranged between

1.15 – 4.27) four weeks after aflatoxicosis and then retained to 4.46 mg/ml (ranged between 3.07 –5.13) six weeks after the aflatoxicosis.

In TRT group the HSC transplantation elevated the means of IgG level significantly ($P < 0.05$) from 5.08 mg/ml (ranged between 1.98 –7.18) in zero day and 5.87 mg/ml (ranged between 3.55 – 7.79) two weeks after exposure to AF to 13.77 mg/ml (ranged between 6.45 –15.86) and 7.79 mg/ml (ranged between 2.66 – 10.46) four and six weeks after the aflatoxicosis, respectively.

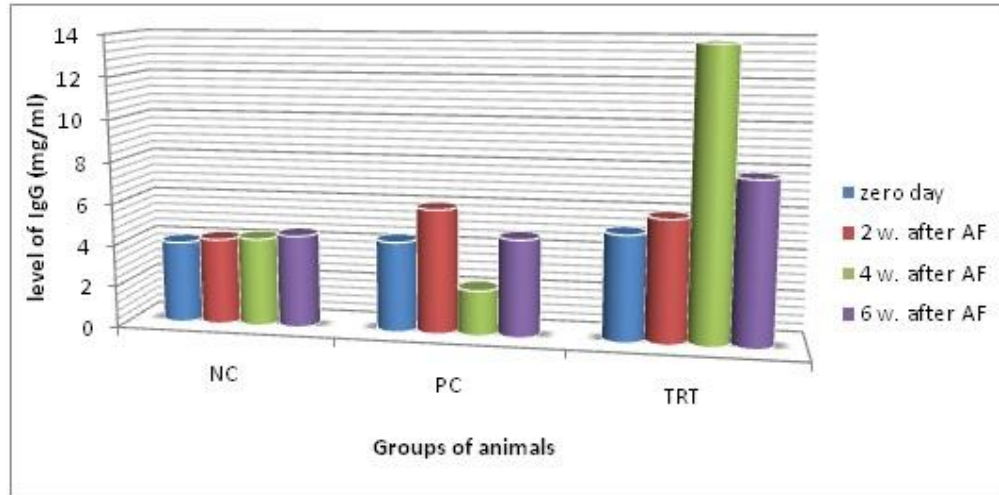


FIGURE 4. Histogram of means of IgG

C) IgM

The levels of IgM were not affected in any group, where in NC group the means of IgM were 184.89 $\mu\text{g/ml}$ (ranged between 77–193) ; 181.33 $\mu\text{g/ml}$ (ranged between 102 – 254) ; 216.83 $\mu\text{g/ml}$ (ranged between 172 – 286) and 307.75 $\mu\text{g/ml}$ (ranged between 225 –389) in the four consecutive periods, respectively, so in the PC group the means were 167.21 $\mu\text{g/ml}$ (ranged between 113–244) ; 154.6 $\mu\text{g/ml}$ (ranged between 124 –215) ; 195.92 $\mu\text{g/ml}$ (ranged between 87

– 235) and 245.63 $\mu\text{g/ml}$ (ranged between 167 – 385) in zero day, two , four and six weeks after aflatoxicosis, respectively. In TRT group , the means of IgM also remained without significant differences ($P < 0.05$) from 159.83 $\mu\text{g/ml}$ (ranged between 97 – 202) in zero day to 164.25 $\mu\text{g/ml}$ (ranged between 87 – 305) ; 154.86 $\mu\text{g/ml}$ (ranged between 73 – 266) and 213.67 $\mu\text{g/ml}$ (ranged between 154 – 317) in two , four and six weeks after aflatoxicosis, respectively.

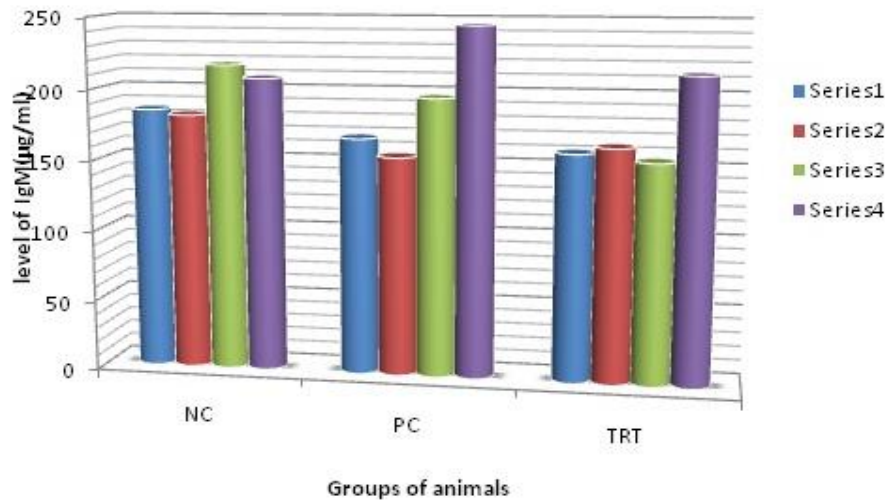


FIGURE 5. Histogram of means of IgM

DISCUSSION

The results of LTA revealed markedly the immunotoxic effects of AF in PC group, and the protective and therapeutic roles of HSC to overcome the immunosuppression induced by AF, in TRT group. The immunotoxic effects of AF which appeared in PC group are in agreement with those of Pier (1992); Van Heugten *et al.* (1994) ; Silvotti (1997) , Fernandez *et al.* (2000) and Dhanasekaran *et al.* (2011) who found prominent effects of AF on phagocytic activity and cellular immune functions. On the other hand , disappearance or recovery of the immunosuppressive effects of AF in the ewes of TRT group who treated with HSC soon after course of AF exposure suggests the therapeutic ability of HSC to overcome the immune suppression resulted from aflatoxicosis in sheep , and this is in agreement with Porta *et al.* (2008) who used the HSC transplantation to treat the SCID and other severe immunodeficiencies ; as well as , with Shiruz *et al.* (2005) who suggested that HSC regenerate an entire immune system comprising dendritic cells, tissue macrophages, B-cells, T-cells and natural killer (NK) cells. In addition to Lymphocyte transformation assay , which used for estimation of both cellular and humoral immunity , The cellular immunity was measured through using an inexpensive, easily applied DTH test using PHA as a mitogen, which become a popular, effective test that can be applied with minimal training , without specialized equipment, applicable to large numbers of animals, can assess all the cells and mediators involved in the response, and requires no presensitization (Jacobs *et al.*, 1981; Smits *et al.*, 1999). The DTH test provides a measure of the proliferative response potential of circulating T lymphocytes to an injected PHA has long been recognized for its mitogenic and blastogenic properties (Hungerford *et al.* 1959). Jacobs *et al.* (1981) stated that PHA acted as a primary irritant initially and resulted in the supportive phase which decreased with time and was eventually replaced by the mononuclear infiltrate seen at 72 hours. The highest increment in the skin fold thickness in this study was observed predominantly 24 hour after injection of the PHA. These results of the DTH test in PC group were in agreement with Fernandez *et al.* (1997) who observed decrease in cellular immune responses in lambs with aflatoxicosis which demonstrated by the intradermal test using PHA, also the same result was reported Richard *et al.* (1983) in steers and by other researchers in other animal species and this effect has been observed as a low response to intradermal tests (Giambone *et al.*, 1978 ; Raisuddin *et al.*, 1993; Van Heugten *et al.*, 1994), This response is due to the toxic effects of the AFs on T lymphocytes , while in TRT group these toxic effects were not occurred, where the ratios of increased skin thickness remained in high levels in compare with those of zero day and of PC group, and this result agreed with results of Jacobs *et al.* (1981) who had 116% , 136% and 199% of increment at different doses of PHA.

The high ratios of increment skin thickness in this study indicate the preventing and therapeutic roles of HSC in ewes in TRT group, where the HSC offered a compensatory resource of lymphocytes instead of those lost due to AF until their effects were diminished or ended.

This result is in agreement with suggestion of Fernandes *et al.* (2011) who stated that HSC transplantation has been the only curative treatment for a large variety of immune deficiency diseases. The response to intradermal injection of PHA four weeks after the last dose of AF was retained to nearby the ratio of the zero day , so this result was not differed from those of Fernandez *et al.* (1997) who found no effect during the clearance period of 35 days, indicating a recovery of the toxic effects on cellular immunity after the withdrawal of the toxic feed. Levels of immunoglobulins have a different degrees of affection , while levels of IgA not affected significantly ($P < 0.05$) in NC and PC groups, they increased significantly ($P < 0.05$) two weeks after aflatoxicosis in PC group and two and four weeks after aflatoxicosis in TRT group, so this result have agreement with Pier *et al.* (1985) and it is attributed to the demand of the mucous surfaces of the digestive tract to this immunoglobulin where it play an important protection role (Quinn *et al.*, 2004) , thus the HSC play an important role in protecting them from further damages resulted from AF or from the secondary bacterial infections where *E. coli* was isolated from 3 diarrheic ewes in PC group and from one diarrheic ewe in TRT group, and *Salmonella* spp. also isolated from an ewe in PC group. While the level of IgG decreased significantly ($P < 0.05$) two weeks after exposure to AF in PC group , it increased significantly ($P < 0.05$) in TRT group to bind to the receptors on phagocytic cells thereby facilitating destruction of the antigens by intracellular killing (Quinn *et al.*, 2004). Levels of IgM were not affected by AF in any group , this in agreement with Pier *et al.* (1985) where this immunoglobulin specialized to acute infections , while the AF in this study used for 14 consecutive days which is tend to chronicity.

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