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Effect of Trichothececns toxin on stem cells isolated from Umbilical cord blood

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Abstract: The study included the collection of umbilical cords blood samples immediately after delivery . Hematopoietic stem cells were isolated from the umbilical cord blood by the gradient density method. The hematopoietic stem cells (MNCs) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10 % of fetal calf serum, then subjected to *in vitro* study to confirm the toxic effects of Trichothecenss extracts with the following concentrations (0.001,0.0039,0.0078, 0.0156, 0.031, 0.062, 0.13, 0.25, and 1 μg/ ml) and to detect the inhibition rate of hematopoietic stem cells by using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. The cytotoxicity assay (*in vitro* study), revealed that the inhibition rate increases seriously with the increase of Trichothecens concentration. Hence the concentration 1 μg /ml has given inhibition rate which reached 100%, while the concentration 0.001 μg /ml did not eliminates any type of cells. Trichothecens has a highly toxic effect on human hematopoietic stem cells, and its concentration is high and positively associated with the rate of cell death increases. Furthermore, the concentration 1 μg /ml of Trichothecens extracted from the Trichothecensgenic isolates can kill 100% of hematopoietic stem cell.

Key words: MNCS , In vitro, Trichothececns , Cytotoxicity.

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Introduction:

Trichothecenes are a very large family of chemically related mycotoxins produced by various species of *Fusarium*, the most important structural features causing the biological activities of trichothecenes are: the 12,13-epoxy ring, the presence of hydroxyl or acetyl groups at appropriate positions on the trichothecene nucleus and the structure and position of the side-chain. They are produced on many different grains like wheat, oats or maize by various *Fusarium* species such as *F. graminearum* (1).

Stem cells are unspecialized cells that develop into the specialized cells that make up the different types of tissue in the human body. They are characterized by the ability to renew themselves through mitotic cell division and differentiating into a diverse range of specialized cell types. They are vital to the development, growth, maintenance, and repair of our brains, bones, muscles, nerves, blood, skin, and other organs .Stem cells are found in all of us, from the early stages of human development to the end of life (2).

In 2005, scientists at Kingston University in England were purported to have found another category of stem cells. These were named cord blood embryonic-like stem cells, which originate in umbilical cord blood. Korean researcher Hwang Woo-Suk (2004–2005) claimed to have created several human embryonic stem cell

lines from unfertilised human oocytes(3).

Scientists at Newcastle University in England create the first ever artificial liver cells using umbilical cord blood stem cells in October 2006. It is suggested that these stem cells have the ability to differentiate into more cell types than adult stem cells, opening up greater possibilities for cell-based therapies. Then, in early 2007, researchers led by Dr. Anthony Atala claimed that a new type of stem cell had been isolated in amniotic fluid (4). Umbilical cord blood (UCB) is the blood that is found in the placenta and the baby's attached umbilical cord after its birth (5). Cord blood is used because the stem cells can be found in it, and the latter component is used to treat genetic and hematopoietic disorders. UCB is considered to be the foundation of the scarce but priceless (6). Primary HSCs and progenitor cells that are responsible for repeating the system of hematopoietic that related to the patients with malignant and nonmalignant disorders cured with myeloablative treatment (7).

Reported studies on the first hematopoietic cell transplant in which UCB was used instead of Bone marrow (BM) as the source of hematopoietic cells. They were able to reconstitute the hematopoietic system of a child with Fanconi anemia by means of UCB from an (Human leukemia Antigen) HLA-identical sibling (8, 9). The concentration of HSCs in UCB collections is the same to those in collections of BM for transplantation (10).

Human UCB also contains a more primitive subpopulation of mesenchymal stem cells (MSCs) than adult BM whose immature cells express the adhesion molecules such as CD13, CD29, CD44, CD90, CD95, CD105 and MHC class, but not the antigens of hematopoietic differentiation such as CD34 (11,12). Thus, it appears that the differentiation of UCB stem cells progenitors might constitute an alternative strategy for cellular therapies of diverse disorders.

Materials and Methods:

Cord blood collection:

Umbilical cord blood (UCB) was obtained from 12 women of age 20 – 35 years of caesarean section and Natural Birth in Woman and Children Hospital in AL-Anbar. The protocol was approved by the local hospital ethics committee, and the mother's consent was obtained before cord blood was taken. Cord blood was collected before placental detachment, where the placenta was still in the uterus. An addition to that, the cord blood was collected after placental detachment from the women.

All UCB specimens were freshly collected from the umbilical vein. Immediately after delivery of the baby, the umbilical cord clamped then breaking the link between the baby and placenta, the baby separation of the cord, and the cord, then cleaned a 5 – 8 cm area of umbilical cord with antiseptic solution and, in open method, the blood bag needle was inserted in the umbilical cord vein.

The blood was flow by gravity into the bag containing citrate phosphate dextrose adenine-1 (CPDA-1) anticoagulant approximately 25 ml, since total collections was approximately 100-120 ml. During collection the blood bag was shaken gently, so that the anticoagulant freely

mixed with UCB (13) While , by the closed method, the blood was collected directly by insert the venoject needle into the umbilical vein, so the blood was drown inside the vacutainer tubes which supplied with heparin as anticoagulant. The UCB samples were handled precisely and brought to the stem cell culture laboratory to avoiding the direct sunlight exposure and the high extreme temperature.

Cord blood cell separation:

After a successful collection of UCB, it was kept in an anticoagulant treated bag and kept at 4°C and processed within few hours.

The mononuclear cells (MNCs) were separated from UCB by gradient density centrifugation according to the protocol described by(14) as follows:

1- UCB was diluted in a proportion of 1:1 in a PBS.
2- Four ml of diluted blood was transferred to 10 ml round bottom tubes and layered carefully on 3 ml of Ficoll-Paque solute and centrifuged at 2200 rpm for 25 min at 4°C in order to isolate PBS (Figure- 1).

Figure (1): Diluted blood overlaid on Ficoll-Paque before gradient centrifugation

3- Centrifugation was resulted in four distinct layers (from top to bottom):
   - A transparent yellowish plasma layer.
   - A cloudy buffy coat layer containing the mononuclear cells.
   - A clear layer of Ficoll.
   - A red layer containing red blood cells and polymorphonuclear cells (15).
4- By using Pasteur pipette, the MNCs rich zone (buffy coat layer) was aspirated and transferred into a new 10 ml round bottom tube and washed twice with culture medium (DMEM) through centrifugation at 4°C in 2000 rpm for 8 minutes and 1000 rpm for 10 minutes, respectively.
5- The final pellet was resuspended with 1ml of culture medium supplemented with 10% FCS and was considered ready for cells count, viability and percentage of HSCs by taken 100 µL from these suspended cells, while the remainder was subjected to cultivation and isolation of the MNCs (hematopoietic stem cells) (figure-2).

Figure (2): Separation tube of blood four layers after gradient centrifugation, the upper one, represent the supernatant plasma, the medium cloudy layer (Buffy coat), the HSCs, the layer of the Ficoll-Paque and the remainder cells which settled in the lower layer.

Determination of cell number and viability:

The cell count and viability can be determined by using trypan blue stain. About 100 μL from the resuspended cells were diluted 1:1 with trypan blue solution and transferred into an Eppendorf tube then incubated at room temperature for two minutes to determine the cell viability, where dead cells were stained (blue colour), while alive cells not stained (16).

Then 10 ml of the mixture was transferred to a haemocytometer chamber. Viable cells in each of the four corners squares on either sides of the counter chamber were calculated. The average of the counts was multiplied by 2×10^4 to give the number of cell / ml (17).

Viable Cells (%) = [No. of viable cells/Total No. of cells (dead and viable)]× 100

Isolation and cultivation of the hematopoietic stem cells from the human umbilical cord blood:

After determination of the cell count and viability the remainder cells were subjected to isolation and cultivation procedure according to (18) as follows:

1. A number of 1×10^6 cells were cultured in a 25-mm tissue culture flask contain 5 ml of DMEM medium supplemented with 10% FCS and 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B and Plated onto a 25-mm tissue culture dish and incubate at 37°C, 5% CO2 overnight.
2. For 3 consecutive days, the nonadherent cells were replated onto a new tissue culture flask to remove any contaminating adherent cells, (where it is known that the nonadherent cells are the MNCs).
3. After 3 days, the nonadherent cells were harvested by centrifugation (10 min, 300 rpm), the supernatant was aspirated and were cultured again in similar condition.
4. The cells monitored daily and the half of the medium was replaced if the color turns orange until the cells reached to 80 % monolayer confluence.

Measurement of the Viable Hematopoietic stem cells by MTT Assay (17):

1. An aliquot of 100 μl of the cell suspension was implanted in each of the 96 well microtiter plate, (104 cell /well). The plate was incubated at least for two hours in a CO2 incubator.

2. Serial of concentrations from each purified extract (Trichothecenes toxin) was prepared from each stock solution to get (1, 0.5, 0.25, 0.12, 0.063, 0.031 0.016 and 0.00781) µg / ml, then sterilized with 0.22 µm millipore filter.
3. Then 100 µl from each concentration of the previous Trichothecenes toxin was added to each well of the lymphocytes implanting plate.
4. The plate was incubated at 37 ºC in a CO2 incubator for 24 hours.
5. Eventually, 50 µl of MTT stain (2mg/ml) was added to each well and then incubated for a further 4 hours.
6. After centrifugation, the medium (DMEM) was removed gently by fine gauge needle. Then MTT-formazan crystals that formed only as a result of live cells were dissolved in 100 µl of DMSO and added to all wells.
7. Absorbance at 620 nm was recorded immediately by ELISA reader.
8. Viable cell Lymphocytes as a percentage was calculated as followed:

   [Absorbance of the test /Absorbance of negative control] X 100.

9. A comparison between the results of both extract (Trichothecenes) at different concentrations was statistically calculated to pick up the most effective dosages of each concentration that may cause lymphocytes killing.

Results and Discussion:

Stem cells are cells derived from blood, bone marrow or embryonic cord blood, these cells have the ability to give other types of cells in addition to blood cells. Some studies have shown the development of nerve cells from stem cells, and can summarize blood cells isolated from umbilical cord blood .The secret as single cells can be used in patients who have a difficult match between them with the possibility of tissue rejection or less likely to transmit viruses (19).

The objective of this study was to know the fact that clinical results of transplantation HSCs depend mainly on the number of nucleated cells transplanted.

For reasons of homogeneity and comparison between the type of birth, samples were collected from births Caesarean birth and natural birth , Only blood from the umbilical cord vein was collected , not from the placenta. Gravity and natural movement were used to drain, the system was easy quick and unpollutedlly during the cord blood colaction process.

The blood was not collected after the exit of the placenta to obtain hemorrhagic foci in the delivered placentas well as the presence of clots in the placentas fetal vessels and this in turn leads to a decrease blood flow component of the umbilical cord (20) , 20 ml of umbilical cord blood was collected and the result was a high blood volume , The reason for this was the compression of the placenta as a result of uterine contractions as a result, the blood flows into the sterile blood bag, and this result is consistent with what is found was (21).

Once sample collection was complete, the unit was transported back to the laboratory and stored at room temperature until processing was initiated, there should be variation among banks and among collection sites for the same cord blood bank. A large infection can occur in of the mononuclear cell layeras a result of

containing umbilical cord blood on other cells, including red blood cells. Other steps should be added to the process of isolating the red blood cells, leading to removal of RBCs minimizes background assay. The colonies of blood components can be calculated by optical microscopy (22).

Trypan blue stain dye was used in the observation of effective cells if non-pigmented living cells were observed in blue and looked bright while noting the blue color visible on the dead cells, afterwards, mononuclear cell were seeded and used for toxicity testing using Trichothecenes toxin.

The HSCs suspended in the tissue culture during three days of culture, the suspended cells (HSCs) appeared as a homogenous population of MNCs characterized by round shape and high nucleus to cytoplasm ratio. Three days later, after centrifugation, 50% of the medium was changed; MNC cells began to form a cluster 3-5 days after the revealed cultivation. One week later, the HSC formed about 80% confluence. At this time the culture medium was discarded after centrifugation and the cells washed with PBS, and passed into a new flask with the same cultivation conditions and so on until the cells were seeded into a microwell plate at a density of $2x10^4$ cells/well and cultured in 200lm DMEM supplemented with 10% FCS of each wells, then incubated until they are treated with different concentrations of Trichothecenes (Cytotoxicity assay). (Figure-3 and Figure-4).

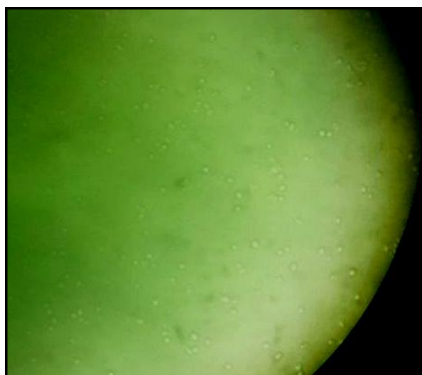

Figure (3): Morphology of MNC isolated by the gradient centrifugation, which cultured in DMEM revealed by inverted microscope. Maximized appearance of the cells 24 hours after cultivation.

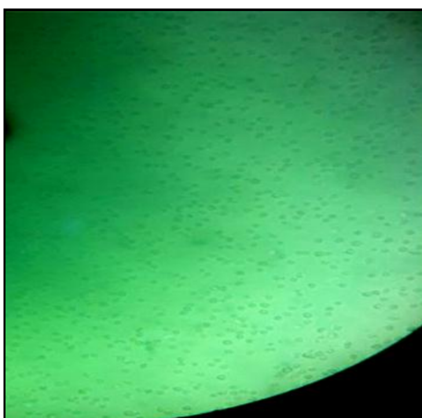

Figure (4): Morphology of MNC isolated by the gradient centrifugation, which cultured in DMEM revealed by inverted microscope. Maximized appearance of the cells 2 weeks after cultivation.

Cytotoxic Effects of Trichothecene:

The study concentrations of (39%, 69.50%, 72.3%) from trichothecene toxin (toxin 1,toxin2, toxin3) and 100 % standard , As for the fourth toxin only gave its value very little concentration and thus neglected its outcome, gave different rates mean of inhibition reached to (90.22, 103.46, 45.92, 56.08) respectively.

The relationship between the number of living cells and the concentration of toxin was estimated. Linear, quadratic and causal functions were used to express the relationship, The linear relationship was the best according to F test. The relationship indicates that the number of living cells when the concentration toxin 1 µg /ml is zero, the number of living cells is quite large and decreases as the concentration increases. This indicates that the concentration increases by 10%. The number of living cells decreases by (5.5), meaning that the change in concentration stands behind 50% Living cells. This is due to the fact that trichothecenec has the ability to dissolve or break down DNA the human lymphocytes and shown to inhibit mitogenic stimulation of human lymphocytes, this result came from what he found (23) he showed that both the growth inhibition and DNA damage experienced by the human lymphocytes increased linearly with increasing concentrations of toxin.

In the case of toxin II was the best estimated function is the cubism and indicates that the number of living cells when the concentration of zero is 111.56 and then decreases this number increasing emphasis, as it decreases initially in a decreasing manner and then begin decreasing the increase in high concentrations Thus, the coefficient of R^2- determination indicates that the change in concentration of toxin is 88% of the changes in the number of living cells (Figure 6). It is also possible to note the strong toxicity of the trichothecens toxin and its effect directly on the immune system, which is considered to be the main cause of this toxin and the effect of T-2 on cellular and humoral responses in gut-associated lymphoid tissue. This is acceptable as I noticed (24). The effect of small dose of the toxin T-2 TOXIN will result in chronic infection.

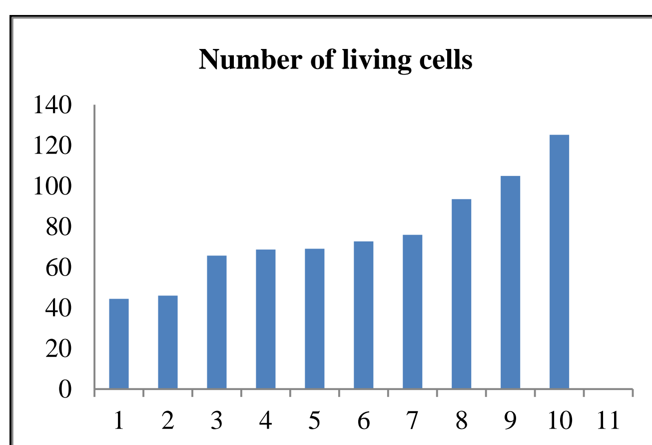

Figure (5): Inhibition rate of MNC exposed to in Toxin 1

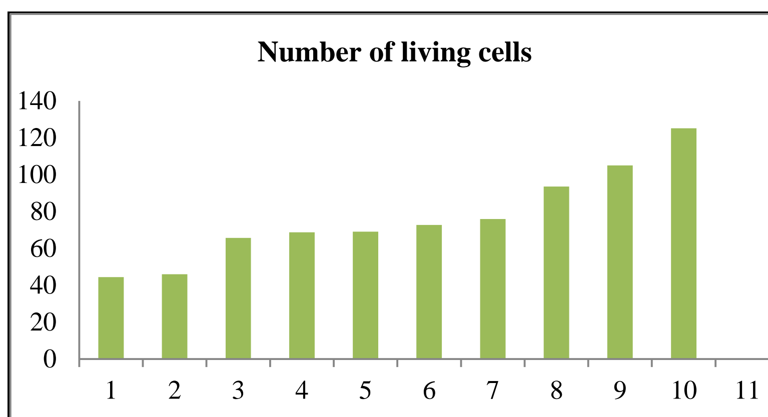

Figure (6): Inhibition rate of MNC exposed to in Toxin 2

While the relationship between the living cells and the concentration of the third toxin was confirmed. The linear function showed its superiority in terms of the significance of the function in the expression of the relationship between the living cells and the concentration of the poison as it reached about 44. 69 when the concentration of the poison from zero and then the less the concentration wich increased by 2. 8 the greater the concentration by 10% Thus, the concentration of toxin changes to about 66% of the changes in the number of living cells (Figure 7).

This is due to the ability of the toxin to inhibit the processes of protein synthesis, which in turn affects DNA RNA as well as preventing active dividing cells by dividing rapidly such cells as the lining of the digestive tract, skin cells and lymphocytes, adding that it causes severe acute and chronic infections, which in turn will lead to deterioration of human health (25).

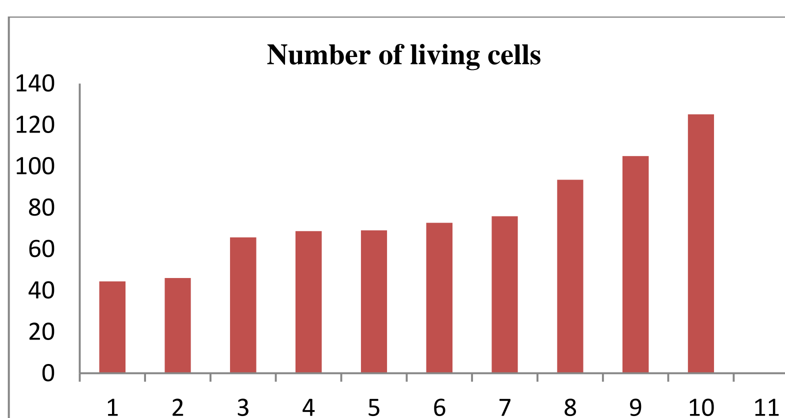

Figure (7): Inhibition rate of MNC exposed to in Toxin 3

As for the relationship between the number of living cells and the concentration of the standard, the linear relationship was also the best in terms of the F value of the table and the moral parameters of the function and the coefficient of the selection of the function The changes indicated that the number of living cells approaching 58 in case the type of poison is standard and its concentration is close to zero while increasing This number in the case of the first toxin by about 28 to become about 28 to become about 86.

In the case of toxin II, it increases by 35.5% to 93.5%, whereas in the case of toxin III it decreases by about 9.5% to about 48.5, and the number of cells decreases as the concentration increases. As the concentration increases, it decreases by about 4.7%. (Figure 8). This is due to the large effect of the toxity of the Trichothececns and its effect directly on the plasma membrane of the cells leading to the exit of its contents and thus will lead to sudden death, the Apoptosis of the effects of toxicity and that through its effect on organs and tissues may affect factors or environmental effects leading to death, indicating the inevitable toxicity (26,27).

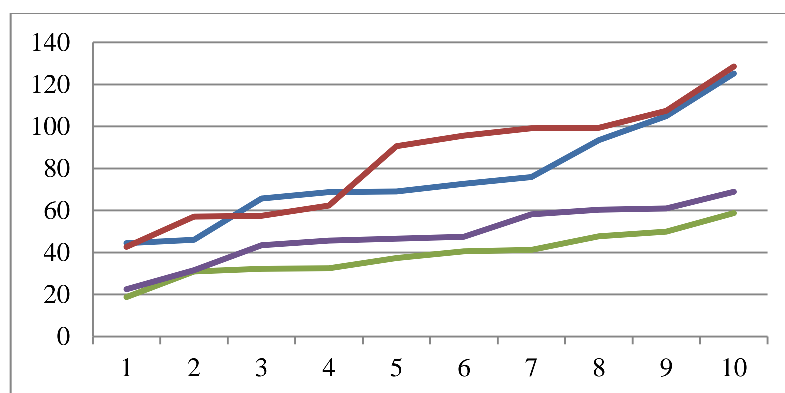

Figure (8): Inhibition rate of MNC exposed to Trichothecenes which measured via cytotoxicity assay

The effect of the type of fungal toxins on the course of the disease was estimated by giving variables to examine the type of toxins on the slope and proved that there were no significant differences, while a significant presence was observed only in the average number of living cells by type of poison, note significant differences between the types of toxins. It is clear from the (Table-1) that there are differences between the toxins, which reached the highest mean in the first and second poison by a very significant difference according to the test t, which reached the value of 3.148, followed by the standard with a very significant difference between it and the no-poison and the difference between them 5.56 Thus we can conclude that the concentration effective effect on production the toxin. The presence of aflatoxins and toxins produced by *Fusarium* fungi has been closely observed despite many biochemical and biological studies that have demonstrated each toxicity (28) This result came in agreement with what he found(29) as it observed a significant reduction in the number of lymphocytes when the toxin concentration was high , The reason for the low activity of these cells to affect the mitochondria, which leads to their weakness and reduced their ability to live (30).

Table (1): Shows the test of the difference between the production of toxins

Mean	Toxin 1	Toxin 2	Toxin 3	Standard
Toxin 1	-	3.148**-	7.656**	5.56**
Toxin 2	3.148**	-	21.817**	15.5**
Toxin 3	7.656**-	21.817**-	-	7.99**-
Stan.	5.56**-	15.5**-	7.99**	-

It is clear from (Table -2) that the high concentration and increase of more than 50% leads to the reduction of all toxins, and the values of test T showed a very significant difference between concentrations that are equal to or more than 50% compared to the lowest concentrations.

The higher concentrations are about 45.22, while rising at the reduction to concentrations less than 50% to about 84.46 and the test of its role in the difference between the averages and estimated 5, which is very significant for the rest of the averages .In the same way, the concentrations of less than 0.125 are different from those exceeding the table (4.16), showing the average production of the toxin according to the two levels. The morphological changes observed in lymphocytes affected by fungal toxins are due to the direct effect on DNA, resulting in a chromatin crash and eventually lead to cell death. Another cause of cell death can be seen as the reaction of various types of fungal toxins, this natural interaction may be highly complex to the very leading to toxic effects and its carcinoma. (31 ,32) Table (2).

Table (2) shows the average production of poison by concentration level and T test values for the extent of moral difference

Con.	Toxin 1	Toxin 2	Toxin 3	Sta.
0.25≥	45.22	49.88	24.8	2.7
0.25≥	84.46	92.57	42.5	53.95
T. test	**5.158-**	**3.916-**	**2.56-**	**4.805-**

Conclusions:

1. The toxicity of trichothecens is highly toxic to the stem cells of the blood. This leads to an increase in cell death rates the greater the concentration of toxin.
2. The process of collecting blood and placenta in the uterus is the best way to reduce the pollution.
3. The concentration 1 μg /ml of trichothecens extracted from the trichothecengenic isolates which can kill 100% of hematopoietic stem cells.

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