

Evaluation for the Effect of Heat Stable Enterotoxin (a) Produced by Enterotoxigenic *Escherichia coli* on Different Cancer Cells *In Vitro*

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Date of acceptance 1/3 / 2010

Abstract

This study was conducted for evaluating the cytotoxic effect of heat stable enterotoxin a (STa) produced by enterotoxigenic *Escherichia coli* on the proliferation of primary cancer cell cultures, obtained from tumor samples that were collected from (13) cancer patients and as follows: (five colon cancer patients, two bladder cancer patients, two breast cancer patients, two stomach cancer patients and two lung cancer patients), and on normal cell line (rat embryonic fibroblast / REF) (*in vitro*) with the use of different concentrations starting from (1) mg/ml and ending with (0.0002) mg/ml by making two fold serial dilutions by using the 96- well microtiter plate, and in comparison with negative (PBS) and positive (MMC, at concentration of 10 µg/ml) controls .

Results showed that, after (24) hours of exposure to STa, the growth of all primary cancer cell cultures obtained from colon cancer patients was inhibited by STa treatment and this inhibition was concentration dependent. Also it was shown that the cytotoxic effect of the high concentration of STa was close to that seen after MMC treatment. While no differences were seen in the growth of all primary cancer cell cultures that were obtained from the other cancer patients, which mean that STa treatment neither inhibit nor enhanced their growth. At the same time STa did not show or has any cytotoxic effect on the normal cell line (REF).

Key word : heat-stable toxin, *Escherichia coli*, primary cancer cell culture

Introduction

Colorectal cancer is considered to be one of the main causes of death all around the world. Various methods and strategies have been used to treat such kind and others of cancer. The traditional methods include: radiation, chemotherapy and surgery [1]. Recently new approaches have been suggested and developed; one of these using *Escherichia coli* toxin, for the treatment of colorectal cancer [2].

It was found that *E. coli* produces a toxin, which mimics a natural colon process and provoked diarrhea.

However, the toxin also causes a flood of calcium into the affected cells, stopping colorectal cancer cells from replicating rapidly. Unfortunately there is unexplained inverse relationship between the incidence of colorectal cancer and enterotoxigenic *Escherichia coli* (ETEC) infection [3]. The toxin that produced by ETEC, which is heat stable enterotoxin a (STa), that cause one of the serious forms of food poisoning may be used in the treatment of one of the most deadly types of cancer [4, 5].

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Enterotoxigenic *E. coli* (ETEC) is an important cause of bacterial diarrheal illness. Infection with ETEC is the leading cause of traveler's diarrhea and a major cause of diarrheal disease in the undeveloping nations, especially among children [6]. ST is a small monomeric toxin that is closely related to *Shigella* toxins [7]. The heat-stable enterotoxins are a family of closely related peptides. They are classified into two structurally, functionally, and immunogenically unrelated types, namely STa and STb [8]. STa is methanol-soluble and infant mice-active peptide toxin, while STb is methanol-insoluble and active in pigs, but inactive in infant mice. The toxic activity of STa is resistance to protease [9], while that of STb is inactivated by treatment with trypsin [10].

The authors provided a convincing evidence for the presence of a novel intracellular signaling pathways initiated by STa that prevented the proliferation of colorectal cancer cells. Chemically, STa binds to the guanylyl cyclase-C (GC-C) receptors specifically expressed in the intestinal cells. Ligand binding to GC-C activates the intracellular synthesis of the second messenger cyclic guanosine monophosphate (cGMP) [11]. STa hyperactivates this signaling receptor causing large increases in the intracellular cGMP. In fact, GC-C and its ligands have been implicated in the regulation of the balance of proliferation and differentiation along the crypt-to-villus axis in the intestine. As a result, subsequent loss of the initiation of GC-C signaling may represent one key mutational event underlying neoplastic transformation in the colon [12, 13].

However, intestinal GC-C and its downstream intracellular effector molecules are conserved in colorectal cancer. Thus the presence of STa / GC-

C induced cGMP-dependent signaling pathway, through cyclic nucleotide-gated (CNG) channels and calcium was responsible for the anti-proliferation action of STa enterotoxin on human colon cancer cells because control using colon cancer cells devoid of GC-C were without effect [14].

Cancer has been recognized for more than 200 years, it was reported by Hippocrates and Galen. However, AL-Zahrawi was considered as the first physician who described cancer to look a little like crab because of "finger like projection" [15]. Cancer is a disorder of cell growth that leads to invasion and destruction of healthy tissue by abnormal cells. Although cancer has affected human since earliest time, it was a rare disease until the twentieth century. Cancer now ranks second only to heart disease as a major cause of death in the world [16]. Colorectal cancer is a malignant neoplasm of epithelial cell origin affecting the large bowel. It is a common type of malignancies that affect gastrointestinal tract and it is of favorable prognosis provided that, it is diagnosed and treated in an early stage. Cancer detection at an early stage and identifying susceptible individuals can result in reduced mortality from this prevalent disease [17].

In Iraq, at the present time, cancer is considered as one of the most important causes of death especially after the Gulf war in 1991. For this reason, a large number of studies have been carried out on various forms of cancer with a view to understand the biology, diagnosis and treatment of this disease.

This study aims to open up a new approach in the development of anti-cancer drugs. An attempt to provide anti-cancer agent (STa) for the treatment of colorectal cancer with less or no cytotoxic effect on normal cells

and with more cytotoxic effect on cancer cells.

Materials and Methods:

All the chemicals were obtained from Sigma Chemical Co. (USA) and BDH (England).

Solutions for detecting the cytotoxic effect of STa on cell cultures: They were prepared according to the methods used by [18].

Collection of tumor tissue samples: Tumor tissue samples of 5 different types of tumor (colon, stomach, lung, breast and bladder) were collected into sterile tubes containing a transport medium. They were obtained from the operations theatre of Gastroenterology and Hepatology Teaching hospital and Baghdad Teaching hospital.

Detecting the cytotoxic effect of STa on primary cancer cell cultures: it was done according to [18].

a. Management of tumor tissue samples to obtain primary cancer cell cultures:

All the work was done under sterile conditions in the laminar airflow hood as follows: The collected tumor tissue sample was put in a sterile pettry dishes and all the necrotic tissue and fat were removed. Then the tissue was washed at least five times with the culture media. After that tissue was transferred into a sterile tube that contains the culture medium and minced very carefully by a sharp curved seisor, with maintaining the tissue in the culture media. Then the tube was centrifuged at 1000 rpm for 5min, to get rid of the large tissue pieces. Finally, the supernatant was then transferred into another sterile tube and before seeding into a 96-well microtiter plate, the number of the cells must be counted and the number of cells at seeding should be 10^7 cell /ml.

b. Viable cell count: Cells were counted by using trypan blue stain working solution (1:10). Only the dead cells will take up the dye and appear

blue under the microscope while viable cells exclude the dye and appear white, which make it very easy to distinguish dead cells from viable cells, and this done by mixing 0.2ml of cells and 0.2ml of the stain with 1.6ml of PBS. Then after mixing well sufficient volume was aspirated to fill the Neubaur haemocytometer.

c. Cytotoxic assay: 50 μ l of complete culture media was added to each well in the microtiter plate, except the first vertical line. Then 100 μ l of STa(after isolation and purification from bacterial suspension) was added to first well in the first and second vertical line only at a concentration of (1) mg/ml. 6 repetitives were made. Mixed well and then 50 μ l from the first well in the second vertical line was transferred to the second well and two-fold serial dilutions were made until the well number (12). A positive MMC (at a concentration of 10 μ g/ml) and negative PBS controls was added to each well in the seventh and eighth line of the microtiter plate, respectively.

Then 150 μ l from cell suspension (cancer cells suspension), after their counting, was added into each well in the microtiter plate and incubated at 37C° in 5% CO₂ incubator. Later, after 24hr of exposure time, the microtiter plate was removed from the incubator, and 50 μ l of neutral red dye was added, then incubate at 37C° for 2hr. The viable cells will acquire the dye, while the dead cells will not. After that, all the contents of the microtiter plate were removed and the cells were washed with PBS. Then 50 μ l from neutral red extraction solution (ethanol : PBS) at a ratio (1:1) was added. This solution elutes the dye from the viable cells that taken the dye. The absorbency was determined by using the ELISA Reader at 492 nm. All the previous steps were done with all tumor types (colon, stomach, lung, breast and bladder) that were used in

this study. Then the cytotoxic concentration 50 (CC50) was determined by plotting the STa concentrations against the absorbency.

Cytotoxic effect of STa on normal cell lines

The detection of cytotoxic effect of STa on normal cell line was done according to the method of [19]. It was carried out on the normal cell line Ref (Rat embryo fibroblast), at passage (52) that was provided by Dr. Ahmed M. AL-Shamery / Iraqi Center for Cancer and Medical Genetic Research/ Baghdad, and as follows: 2ml of trypsin-versene solution was added to tissue culture bottle (falcon) of 25cm³ that contain the cells (Ref) after the removal of old tissue culture media and washing with PBS, the bottle was shaken smoothly and then inoculated at 37C⁰ for 2min to disaggregate cells and obtain more single cells as much as possible. After that, cells were suspended in a new fresh tissue culture media and counted at a concentration of 10⁵cell/ml by trypan blue. Then 50µl of complete culture media was added to each well in the microtiter plate, except the first vertical line. Later 100µl of STa was added to first well in the first and second vertical line only at a concentration of (1) mg/ml. 6 repetitives were Made. Mixed well and then 50µl from the first well in the second vertical line was transferred to the second well and two-fold serial dilutions were made until the well number (12). A positive MMC (at a concentration of 10µg/ml) and negative PBS controls was added to each well in the seventh and eighth line of the microtiter plate, respectively. Then 150µl from cell suspension, after their counting, was added into each well in the microtiter plate and incubated at 37C⁰ in 5% CO₂ incubator. After 24hr of exposure time, the microtiter plate was removed from the incubator, and 50µl of neutral red

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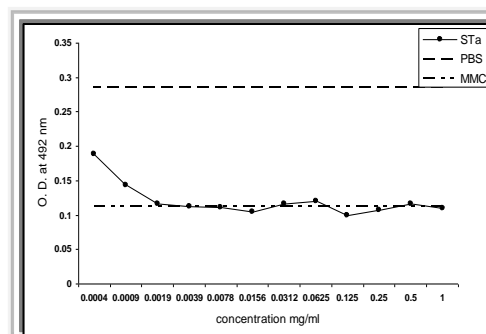
Results and Discussion:

Cytotoxic effect of STa on primary cancer cell cultures. The cytotoxic effect of STa on primary cancer cell cultures was determined by evaluating its effect on: five tumor tissues obtained from patients with colon cancer, two tumor tissues obtained from patients with bladder cancer, two tumor tissues obtained from patients with breast cancer, two tumor tissues obtained from patients with stomach cancer and two tumor tissues obtained from patients with lung cancer.

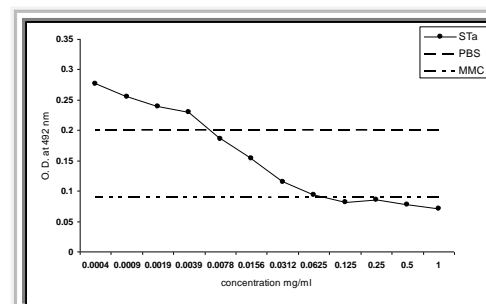
Results of the cytotoxic effect of STa on tumor tissues obtained from five different colon cancer patients showed that STa caused an obvious inhibition in the growth of cancer cells compared with the negative control and this inhibition was close to the inhibition that was seen with the positive control (MMC, at concentration of 10µg/ml), as shown in figure (1/ A, B, C, D and E). These results that associated with growth inhibition of primary colon cancer cell culture after STa treatment were similar to those obtained by many authors [20, 21, 3, 1, 5, 22], and in fact some of them were able to reduce the growth of cancer cell lines with less concentrations of STa. They showed that STa induce colorectal cancer

cytostasis without cell death by targeting guanylyl cyclase-C (GC-C) signaling and the anticancer action of this toxin is mediated by cGMP that dependent influx of Ca^{+2} through the cyclic nucleotide-gated channels. Thus, GC-C is the specific receptor for STa, and without it, STa will be unable to exhibit its cytostasis effect [23, 24, 25, 26]. In fact some chemotherapeutic drugs show their cytotoxic effect by activation of cGMP [27].

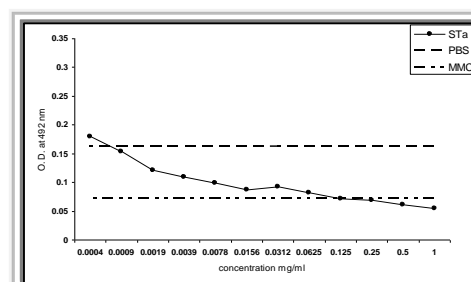
Previous studies showed that STa penchant for intestinal cells only, indicating that, as a drug, it would focus just on these cells and leaves others alone, and upon its injected into the blood it might even specifically combat colon cancer cells that had been migrated to other parts of the body. Thereby, derailing metastasis which is a serious problem in this type of cancer. Pitari and coworkers predicated that even if this toxin only slow the growth of colon cancer cells without killing them, it speculate that it may lead to possible therapy that would control colorectal cancer spread.



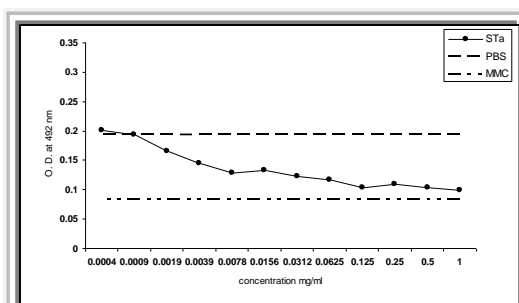
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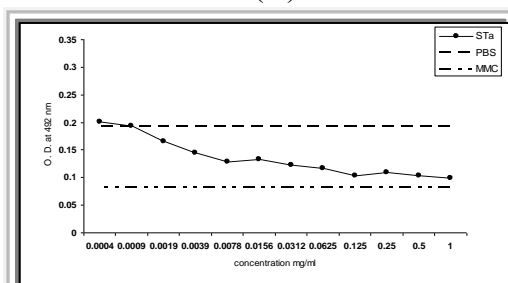
(D)



(E)



(A)



(B)

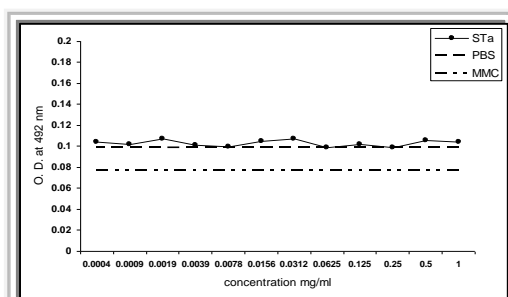
Figure (1): Cytotoxic effect of STa on primary cancer cells cultures obtained from five patients with colon cancer (A, B, C, D, E).

Thus, if the sacrifice for one is to have occasional diarrhea and prevent the tumor in the colon from ever forming or progressing, it worth's it [5].

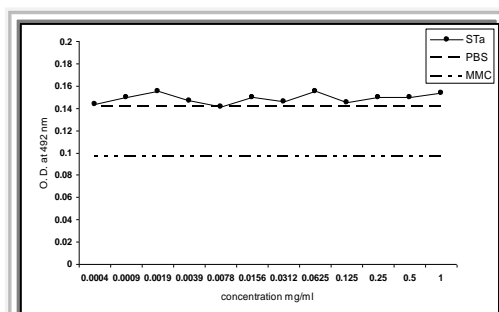
At the same time the cytotoxic effect of STa on primary cancer cell cultures obtained from other organs have been also studied. Results showed that STa had no inhibitory effect on the growth of cancer cells obtained from (bladder, breast, stomach and lung cancer) as shown in figures (2, 3, 4, 5, A and B) respectively.

The most reasonable explanation for the absence of STa effect on these primary cancer cell cultures is that STa does not bind to these cells due to the

absence of the expression of GC-C receptors to which STa binds and exerts its effect [26]. These results were in close agreement with those declared by [28, 29, 30], in which they examined the binding affinity of STa to extraintestinal tissues by using many different types of cancers and with the using of more precise techniques including reverse transcription-polymerase chain reaction (RT-PCR), they showed that STa binding were not detected in all these extraintestinal cancers, and thus no cytostasis effect for STa was detected.

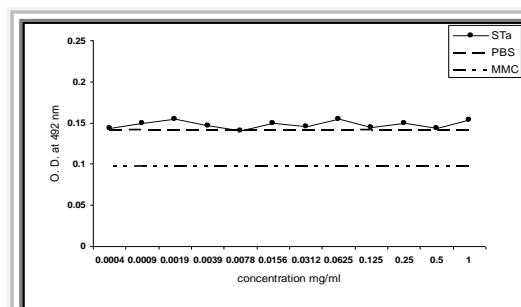


(A)

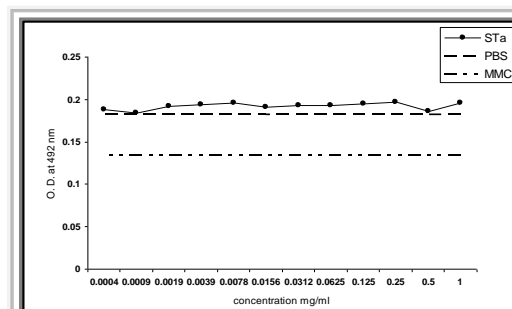


(B)

Figure (2): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with bladder cancer (A, B).

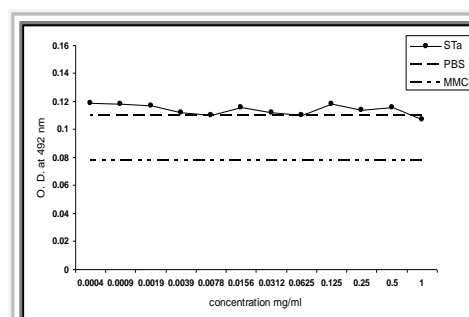


(A)

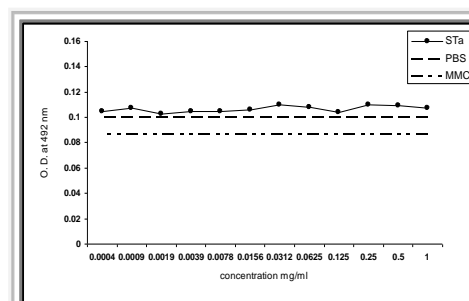


(B)

Figure (3): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with breast cancer (A, B).

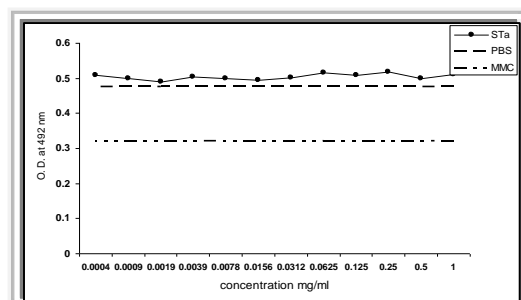


(A)

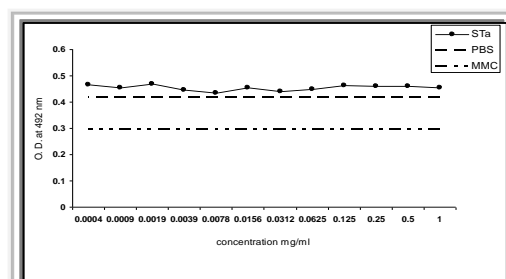


(B)

Figure (4): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with stomach cancer (A, B).



(A)



(B)

Figure (5): Cytotoxic effect of STa on primary cancer cells cultures obtained from two patients with lung cancer (A, B).

Cytotoxic effect of STa on normal cell line (REF): When any substance was suspected to be used in cancer therapy, it must show a selective toxicity on cancer cells but not on normal cells in order to say it is safe. So, when cancer cell lines are used in any study, normal cell lines should be also used as a control for comparison. In this study, REF (rat embryo fibroblast) cell line was used as a normal cell line. Results shown in figure (6) indicated that STa has no cytotoxic effect on normal line (REF) as compared with negative control, and there is an increase in the growth of cells as the STa concentration decreases. This inverse relationship may be explained by the increase in the growth medium concentration at the time of STa concentration decrease, since growth medium concentration plays a significant role in the cell culture technique [18]. Similar results were indicated by [30], who examined the binding and function of STa in normal extraintestinal human tissues and colorectal tumors. They found that STa

was able to bind specifically to all colon and rectum tumors that were examined, while neither STa binding nor STa activation of GC-C was detected in all examined normal extraintestinal tissues. In addition, DNA of tumor cells was found in a relaxed shape, and the DNA molecule was found in an unstable figure because of the far away between the H-bonds which connect the both strands of DNA and this makes it easy for compounds to interfere or associate with both DNA strands. On the other hand, DNA of normal cells has strong H-bonds that connect the both strands to each other and thus making it more stable, so by this way, the compounds can not interfere or associate with DNA strands [31]. However, inhibition of normal cell line growth was detected with MMC treatment at a concentration of (10) $\mu\text{g/ml}$, as compared with the negative control, and this result came in agreement with [32], in which they showed that MMC has toxicity to both normal and tumor cell lines and this toxicity could be increased or enhanced by certain substances.

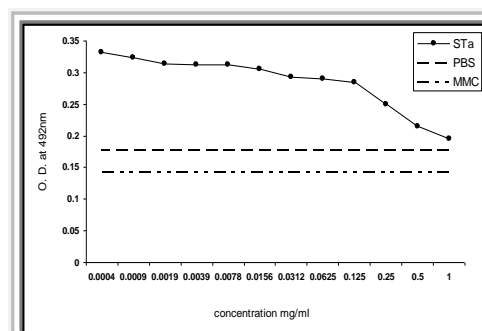


Figure (6): Cytotoxic effect of STa on normal cell line (rat embryo fibroblast / REF).

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تقييم تاثير السم المعوي الثابت حراريا (أ) المنتج من قبل بكتريا ايشيريشيا القولون السمية المعوية على الخلايا السرطانية المختلفة في الزجاج

مها فخري الطائي * محمد رفيق الحديثي ** عبد الواحد باقر الشيباني **

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الخلاصة:

اجريت هذه الدراسة لتقييم التأثير السمي للمعاملة بـ السم المعوي الثابت حراريا (أ) (heat stable enterotoxin a / STa) المنتج من قبل ايشيريشيا القولون السمية المعوية (enterotoxigenic *Escherichia coli*) , على انقسام المزارع الاولية للخلايا السرطانية المأخوذة من عينات ورم تم الحصول عليها من (13) مريضا مصابا بالسرطان وكالاتي: (خمسة مرضى مصابون بسرطان القولون , مريضان مصابان بسرطان المثانة , مريضان مصابان بسرطان الثدي , مريضان مصابان بسرطان المعدة , مريضان مصابان بسرطان الرئة) و ايضا على الخط الخلوي الطبيعي لجنين الجرذ (rat embryo fibroblast / REF) (خارج الجسم) , وباستخدام تراكيز مختلفة من الـ (STa) ابتداء من (1) ملغم/ مل و انتهاء بـ (0.0002) ملغم/ مل عن طريق استخدام التخفيف الثنائية في اطباق الزرع النسيجي ذو 96-حفرة. ولقد تمت هذه الدراسة بالمقارنة مع سيطرة سالبة (دارئ الفوسفات الملحي) و سيطرة موجبة (ميتومايسين (MMC) , عند التركيز (10) مايكروغرام/ مل) .

نتائج هذه الدراسة اشارت الى وجود تثبيط في نمو جميع المزارع الاولية للخلايا السرطانية المأخوذة من المرضى المصابين بسرطان القولون فقط وان شدة التثبيط تزداد بزيادة التركيز. بينما لم يتم تشخيص وجود اي تاثير في نمو المزارع الاولية للخلايا السرطانية المأخوذة من المرضى المصابين بالسرطانات الاخرى وهذا يشير الى ان المعاملة بـ (STa) لم تؤدي الى حدوث تثبيط او تحفيز في نمو هذه المزارع . في نفس الوقت لم يمتلك الـ (STa) او يظهر اي تاثير سمي على خط الخلايا الطبيعي لجنين الجرذ.