Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Development and Preparation of Diphtheria Toxoid by Detoxification of Toxin via Gamma Radiation and Using Modified Semi-Synthetic Medium

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Abstract

Diphtheria is a serious infection caused by *Corynebacterium diphtheriae* that usually attacks the respiratory system. The aim of this study is to produce diphtheria toxin using a modified semi synthetic Linggood medium. Also compare the effect of different doses of gamma radiation (0.5, 2, 5, 10, 15, 25 & 40 KGy) and formaldehyde on detoxifying this toxin. SDS-PAGE, protein content and cytotoxicity of toxoid were evaluated. Some biochemical parameters on serum of immunized mice with diphtheria toxoid were measured. The protein content showed that there was no difference with doses (0.5, 2, 5, 10 KGy) whereas, changed with doses (15, 25 & 40 KGy) when compared with standard toxoid. The gamma irradiated toxoids have a non-toxic effect on Vero cells (ATCC® CCL-81**,** organism: Cercopithecus, Tissue: Kidney). The levels of Urea and Creatinine in serum of all immunized animals groups showed non-significant change compared to control. Meanwhile, activities of serum aspartate aminotransferase (AST) & alanine aminotransferase (ALT) showed no significant difference in case of toxoids irradiated groups (0.5, 2, 5, 10, 15 KGy) while enzymes significantly elevated in toxoid groups (25, 40 KGy and formaldehyde) compared to control animal group. This study revealed that the modified semi-synthetic medium gave a highly purified diphtheria toxin. On the other hand, gamma irradiation with doses (0.5, 2, 5 and 10 KGy) produces safe and effective diphtheria toxoid. It was concluded that this study might be a promising protocol for vaccine production.

Key words: Corynebacterium diphtheriae, diphtheria toxin, fermentation, Vero cell, gamma irradiated toxoids.

Introduction

Diphtheria, a disease of childhood, is a highly infectious life threating bacterial disease caused by toxigenic strains of *Corynebacterium diphtheriae* [1]. The bacterium does not invade the host tissue, but it releases toxin which spreads throughout the host and causes systemic diphtheritic intoxication, it specifically causes the inhibition of protein synthesis and cell death [2]. *C. diphtheriae* usually arises in mucosal areas of the respiratory tract and secretes diphtheria toxin which is spread into body through circulatory system [3]. Despite, the wide spread of diphtheria toxoid which led to a remarkable decrease in the disease, diphtheria is still found as epidemic in developing countries [4], due to low coverage with diphtheria vaccine among children and the large gap of immunity among adults [5]. Nevertheless, diphtheria is still endemic in Eastern Europe, South America, Africa and Southeast Asia, where several thousand cases are reported annually [6]. Nowadays, in Yemen the diphtheria outbreak developed in three epidemic waves, which affected nearly all governorates of Yemen, with 5701 probable cases and 330 deaths from October 2017 to April 2020 [7]. In 2020, a total of 5 countries reported 80 confirmed cases of diphtheria, including 21 deaths in the Region of the Americas. Moreover, the beginning of the COVID-19 pandemic has decreased in the demand for vaccination services, followed by the impact on the vaccination coverages and the delay of vaccination operations [8]. *C. diphtheriae* strain (PW) number (8) was used in this experiment for production of diphtheria toxin due to its low infectivity and high capacity for toxin production in vitro. This strain was recommended by World Health Organization [9]. Diphtheria toxoid is still produced by cultivating the

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Receive Date: 03 June 2022, **Revise Date:** 30 July 2022, **Accept Date:** 04 August 2022, **First Publish Date:** 04 August 2022

DOI: 10.21608/EJCHEM.2022.142616.6231

toxigenic *C. diphtheriae* strains on classic Linggood medium which based on meat digest broth [10]. Although the classic Linggood medium used for *C. diphtheriae* production contains beef enzymatic hydrolysate which gives high toxin yields [11], but unfortunately, this medium has many problems [12, 13]. The classical method is time consuming and cause contaminations due to the many steps in preparation and purification. On the other hand no completely synthetic medium has been proposed to support the growth of *C. diphtheriae* and produce high amounts of diphtheria toxin [14-12]. There was a demand to modify the classic Linggood medium to avoid its disadvantages. Vaccination is disputably the most effective tool to get rid of diseases, billions of animals and people have been saved due to vaccination strategies [15]. Although formaldehyde is the most common chemical used in vaccine industry, which succeeded in preventing several diseases [16], but unfortunately there are several disadvantages of formaldehyde [17-18]. It's worthy to mention that, no vaccine is completely safe or totally effective, their use is supported by their benefit to risk ratio [19-20]. Many researches have focused on the development of vaccine industry, trying to reach to ideal vaccines. Radiobiological techniques are one of these practical applications [21]. Radiation is one of the most promising ways that can be used for this purpose as it can penetrate the pathogens and destroy their genetic material without affecting their surface antigens [18]. For these reasons, the objective of this work is to obtain high yield and more purified diphtheria toxin by using semi synthetic medium and to evaluate the effect of different doses of gamma radiation in detoxifying this toxin.

2. Materials and Methods:

2.1. Microorganism used:

Corynebacterium diphtheriae Park William (PW) strain number 8 was obtained from the Welcome research laboratories code CN 2000, (ATCC No 13812).

2.2. Medium used

2.2.1. Loeffler serum

Loeffler serum consist of 10 gm of glucose was dissolved in 100 ml of nutrient broth then boiled to dissolve completely. Medium was sterilized by autoclaving at 121°C for 15 min. 150 ml of sterile fresh horse serum was warmed to 37°C then added aseptically to cooled medium (45-50°C). A quality control test was done by incubation of plates in the incubator over night as sterility test for the medium **[22].**

2.2.2. Modified semi synthetic Linggood medium

This medium consisted of 10 g beef extract, 0.15 g yeast extract, 1.5 g sodium lactate 60% w/v, 25g maltose and distilled water to make the final volume to 1000 ml. The medium pH was adjusted to 6.8 using 5N NaOH and sterilized by autoclaving at 121 °C for 20 min **[23].**

2.2.2.a. Growth factors

22.5g of $MgSO_4.7H_2O$, 0.115g of ß. alanine, 0.115g of nicotinic acid, 0.0075g of pimelic acid, 1g of $CuSO_4$.5 H2O, 1g of $ZnSO_4.7H_2O$ and 3ml of HCl 35% were dissolved in 100 ml distilled H2O and sterilized by filtration using 0.22 µm stericup Millipore, and 3 ml was added to the cooled sterile semi synthetic Linggood medium **[24]**.

2.2.2.b. 10%Glucose solution

One hundred g of glucose was dissolved in 1 litre of distilled H2O and sterilized by by filtration using 0.22 µm stericup Millipore.

2.3. Inoculum preparation

An ampoule of lyophilized *Corynebacterium diphtheriae* was reconstituted and streaked on Loeffler medium**,** and then a loopful from the overnight grown culture was transferred to Erlenmeyer flasks containing 50 ml of sterile semi synthetic medium which incubated at 35°C for 24 hr. in shaker incubator (140 rpm) (primary inoculum). About 2% from the primary inoculum was added to 200 ml of sterile semi synthetic medium which was incubated under the same conditions for 6 hr.

2.4. Culture (fermentation):

About 2% from the inoculum was inoculated in 2 liters of sterile semi synthetic medium in fermenter (BioFlo3000, 5 L capacity, New Brunswick scientific, USA). The pH was automatically adjusted to 7.8 with sterile 25%NH_4OH and cultivated for 48hr. at 35°C with vigorous shaking (200 rpm). After 18 hr. of cultivation glucose was added (fed batch). The bacterial growth was estimated by taking sample every 2 hr. for measuring the optical density (OD) at absorbance 650 nm to draw growth curve**.** The purity of culture was checked by Gram stain [25] and the diphtheria toxin (Lf) was determined according to Ramon [26].

2.5. Separation and concentration of toxin:

The culture was harvested from late stationary phase and centrifuged at 3000 rpm for 30 minutes (Beckman- J2-21) for clarification. The supernatant is collected and sterilized by filtration using Millipore 0.22 µm stericup. The sterile supernatant is concentrated five-times its original volume using ultra filtration cell (Amicon - Millipore Corporation MA. USA) fitted with a membrane of 10,000 cut off under sterile condition and the supernatant was stored at 4°C [27]. Cell concentration was expressed as dry biomass weight per liter (g/l), by drying the pellet at 60°C for 48 hr. [28].

2.6. Purification using ammonium sulphate precipitation:

The whole toxin was re-precipitated using saturated ammonium sulphate solution with concentration of 20-60% to precipitate whole diphtheria toxin (DT) [29].

2.7. Detoxification of diphtheria toxin using:

2.7.1. Formaldehyde:

The process of detoxification of DT converted by formaldehyde into non-toxic diphtheria toxoid (DTx) was according Glenny and Hopkins [30].

2.7.2. Gamma Radiation:

The process of detoxification of DT into non -toxic diphtheria toxoid (DTx), was as the following, Different doses (0.5, 2, 5, 10, 15, 25 and 40 KGy) of gamma radiation were applied on diphtheria toxin samples, irradiation was carried out at the National Center for Radiation and Technology (NCRRT) using C^{60} gamma radiation source of Indian facility with a dose rate 0.7 KGy / hour at the time of the experiment.

2.8. Evaluation of diphtheria toxoid (DTx):

2.8.1. Protein content determination:

The protein content of DT and DTx was estimated according to Lowry *et al.* [31].

2.8.2. SDS-PAGE:

The purified irradiated toxoids, formaldehyde toxoid and toxin was performed using 12.5% polyacrylamide gel and Mini-Protein III, Electrophoresis cell (BioRad) for determining the molecular weight of protein fractions according to Laemmli [32]. Also a comparison between the classic Linggood which produced by VACSERA and the new semi synthetic one which we improved was performed according to [32].

2.8.3. Cytotoxicity test:

The cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl) 2, 5 diphenyl tetrazolium bromide (MTT assay, sigma –Aldrich, USA) using Vero cell line (ATCC® CCL-81**,** organism: Cercopithecus, Tissue: Kidney) to detect residual toxicity during the detoxification of diphtheria toxin according to **Berridge *et al.*** [33].

2.9. Animals:

Animals tested Inbred BALB/c mice (6-10 week-old and weighing 15-18 g) were used in experimental studies (animal facility of VACSERA) and kept under aseptic conditions at 25°C, 12 hr. /day of light per day, sterilized pelleted food and acidified tap water. BALB/c mice were divided into ten groups (6 mice /group) [34].

2.10. Experimental design:

All groups of mice were injected three times intraperitoneal at two weeks interval (for immunological studies)

The groups were divided as the following:

Group (A) was immunized (i.p) with 200 µl of phosphate buffered saline (PBS) saved as negative control.

Groups (B, C, D, E, F, G and H) were immunized with 200 µl (100 µg/ml) of different irradiated toxoids at 0.5, 2, 5, 10, 15, 25 and 40KGy, respectively.

Group (I) was immunized with 200 µl (100 µg/ml) of formaldehyde toxoid

Group (J) was immunized with 200 µl (100 µg/ml) of standard toxoid {DPT} from WHO as positive control.

Blood was collected from each group of mice seven days post-injection from the mice veins tails [according to the research ethics committee for experimental studies (Hum and animal subject) at National center for research radiation and technology-Egyptian atomic energy authority, (approval No. 27 A/19)].

Sera was collected by centrifugation at 3000 rpm for 15 min. at room temperature and kept at -70°C until used for biochemical analyses.

2.11. Biochemical examinations:

Estimation of quantitative Creatinine using creatinine kit **[35],** Urea levels by Urea kit **[36]** and the activity of serum ALT& AST by using ALT & AST kits respectively according to **IFCC** [37]. All these kits were from Vitro Science

2.12. Statistical analysis:

The data were subjected to one-way ANOVA to evaluate the effect of new toxoid. All statistical analyses were proceeded using the software SPSS, version 21 (SPSS, Richmond, Virginia, USA) as described by **Dytham** [38].

3. Results:

3.1. Seed preparation and culture fermentation:

The Gram smear of *C. diphtheriae* was utilized as indicator for the purity of the culture as represented in **Figure (1).** The results of culture fermentation were described in **Table (1)**, the result showed a gradual increase in the growth and the stationary phase was reached after 48 hr. (the harvesting time) the data represented in **Figure (2),** and limit of flocculation (Lf) value of DT was 60 Lf/ml.

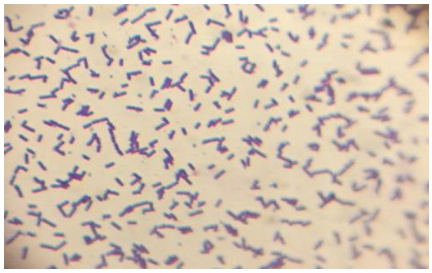

Figure 1: The Gram staining of *Corynebacterium diphtheriae*, the bacteria were identified as Gram positive bacilli

Table1: Summary of experimental results obtained with batch fermentation record

Condition	Record Batch
Optical Density	2.1
Supernatant of culture	1800 ml
Dry cell mass g/L	1.6 g/L

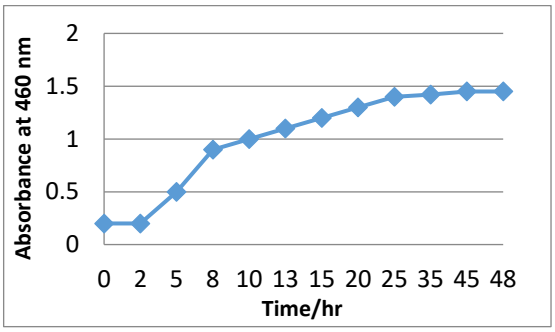

Figure 2: Growth curve of *Corynebacterium diphtheriae*

3.2. Purification of DT:

The DT concentrations in these solutions were determined by Lf test. DT was precipitated between ammonium sulphate concentrations (25% and 34%) as shown in **Figure (3).**

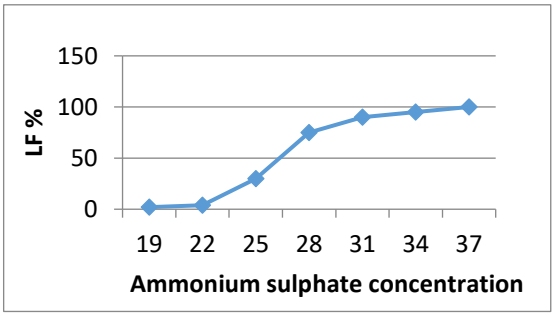

Figure 3: Purification of DT by ammonium sulphate precipitation method, DT was precipitated between ammonium sulphate concentrations (25% and 34%)

3.3. Evaluation of diphtheria toxin and toxoid:

3.3.1. Protein content determination:

The level of protein content for purified DT and DTx showed that there was no difference in the protein content of purified gamma irradiated toxoids with doses (0.5, 2, 5, and 10 KGy and standard toxoid. Whereas, by increasing the dose of radiation to (15, 25 and 40 KGy) the protein content was decreased as compared with standard toxoid the data represented in **Table (2).**

3.3.2 SDS-PAGE:

3.3.2.a Comparison between the modified semi synthetic Linggood medium and classic Linggood medium (VACSERA):

SDS –PAGE illustrated the presence of only purified DT band in case of using the modified semi synthetic Linggood medium while using classic Linggood medium showed DT band in addition to other non-specific bands, as shown in **Figure (4).**

Table 2: The level of protein content for purified DTx

Samples	µg/100µl
Purified 0.5 kGy DTx	240
Purified 2.0 kGy DTx	240
Purified 5.0 kGy DTx	240
Purified 10 kGy DTx	240
Purified 15 kGy DTx	235
Purified 25 kGy DTx	215
Purified 40 kGy DTx	200
Purified formaldehyde DTx	250
Standard DTx	240

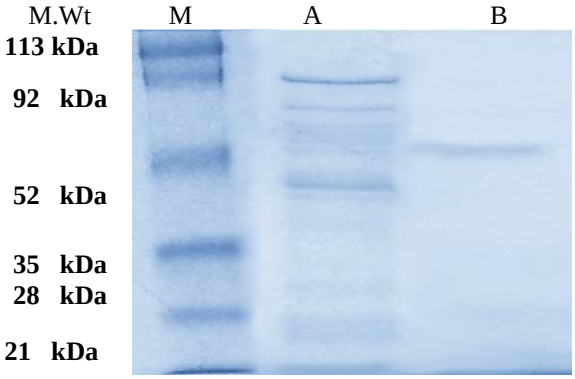

Figure 4: Comparison between modified semi synthetic Linggood medium and classic Linggood medium for DT production using 12.5% SDS-PAGE PAGE.
M: standard protein marker; **Lane A**: classic Linggood medium**; Lane B:** modified semi synthetic Linggood medium.

3.3.2.b. SDS-PAGE after detoxification

The results revealed the presence of purified DTx (58 kDa) in all lanes A, B, C, D, E, F, G, and H for irradiated toxoid (0.5, 2, 5, 10, 15, 25 and 40 KGy) and formaldehyde toxoid respectively as shown in Figure(5).

3.3.3 Cytotoxicity Test:

The results showed that gamma irradiated toxoids with different doses (0.5, 2, 5, 10, 15, 25 and 40 KGy) and formaldehyde toxoid have a non-toxic effect on the Vero cell while the diphtheria toxin showed toxic effect on the Vero cell, as shown in **Figure(6).**

3.4. Biochemical examinations:

The level of urea and creatinine showed no significant difference (P> 0.05) in all toxoid groups compared to control. On the other hand, AST & ALT levels showed a significant difference (P< 0.05) between 25, 40 KGy and formaldehyde toxoid groups when compared to control group but the other tested groups showed no significant difference (P> 0.05) compared to control group, the data represented in **Table (3).**

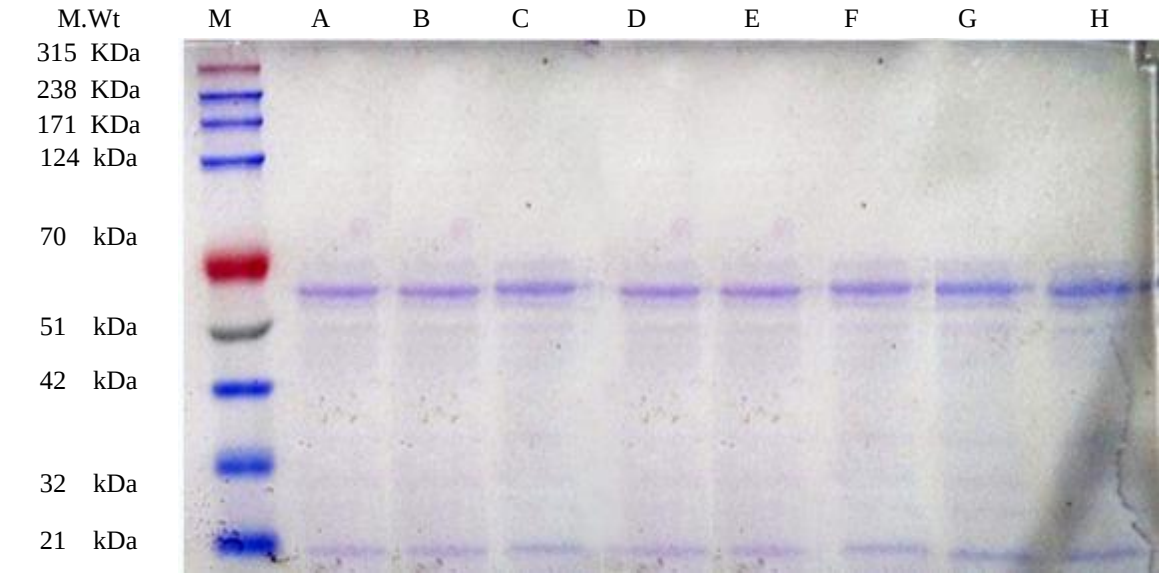

Figure 5: Evaluation of different doses of irradiated diphtheria toxoid and formaldehyde by 12.5% SDS PAGE electrophoresis

M: standard protein marker; **Lane A**: irr DTx 0.5 KGy**; Lane B**: irr DTx 2 KGy; **Lane C**: irr DTx 5 KGy; **Lane D**: irr DTx 10 KGy; **Lane E**: irr DTx 15 KGy; **Lane F:** irr DTx 25 KGy; **Lane G**: irr DTx 40 KGy and **Lane H**: formaldehyde toxoid. **irr DTx =irradiated diphtheria toxoid

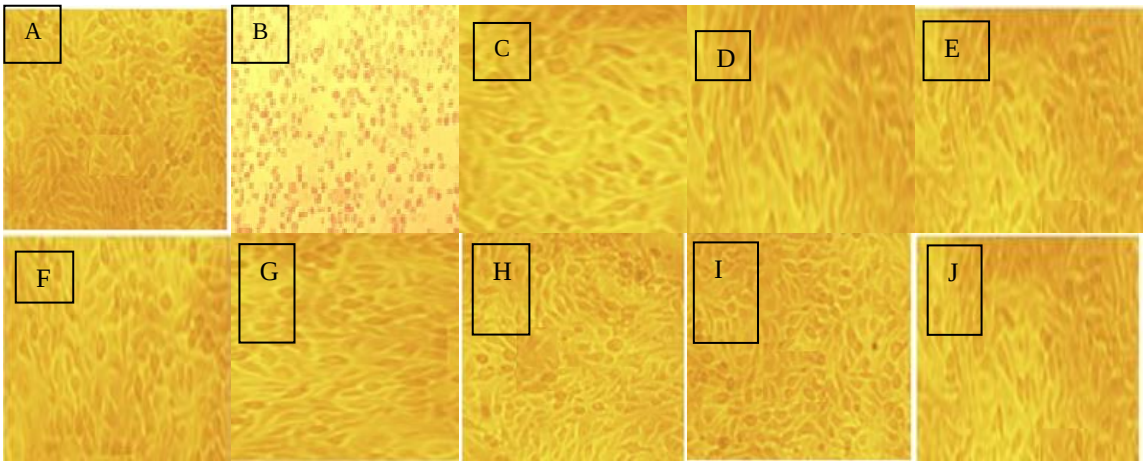

Figure 6: The effect of formaldehyde and different doses of gamma irradiated diphtheria toxoids on Vero cells

A: Normal Vero cells growth after 24 hr (control); **B**: purified diphtheria toxin**; C**: formaldehyde diphtheria toxoid; **D**: irr DTx 0.5 KGy; **E**: irr DTx 2 KGy; **F**: irr DTx 5 KGy; **G:** irr DTx 10 KGy; **H**: irr DTx 15 KGy; **I** irr DTx 25 KGy and **J**: irr DTx 40KGy. **irr DTx =irradiated diphtheria toxoid

Table 3: Blood Urea, Creatinine, AST and ALT levels of BALB/c mice after third injection dose in different groups

Injection groups	Parameters			
	Urea (mg/dl)	Creatinine (mg/dl)	AST(U/L)	ALT(U/L)
Control	36.0±1.4^{a}	0.60±0.09^{a}	60.0±2.2^{ab}	28.2±0.8^{a}
0.5 KGy toxoid	38.1±2.7^{a}	0.67±0.04^{a}	60.6±2.4^{abc}	27.4±1.1^{a}
2 KGy toxoid	37.2±2.4^{a}	0.65±0.06^{a}	60.4±1.9^{abc}	27.2±1.6^{a}
5 KGy toxoid	37.9±2.5^{a}	0.66±0.07^{a}	60.3±1.8^{bc}	27.6±1.1^{a}
10 KGy toxoid	37.4±2.6^{a}	0.63±0.08^{a}	61.8±3.3^{abc}	28.0±1.2^{a}
15 KGy toxoid	36.0±2.9^{a}	0.69±0.09^{a}	59.7±1.7^{ab}	27.8±1.3^{a}
25 KGy toxoid	37.4±2.7^{a}	0.71±0.08^{a}	63.4±2.0^{c}	30.2±1.9^{b}
40 KGy toxoid	36.1±1.9^{a}	0.62±0.08^{a}	70.0±1.5^{d}	35.6±1.1^{c}
Formaldehyde toxoid	38.2±2.3^{a}	0.69±0.04^{a}	93.4±2.1^{e}	41.2±1.3^{d}
Standard diphtheria toxoid	36.4±2.7^{a}	0.62±0.10^{a}	62.8±1.6^{bc}	28.4±1.2^{a}

Means marked with the same superscript letters are non-significantly different (p>0.05), whereas others with different superscript letters are significantly different (p<0.05). Data are average of six replicates; each value represents the mean ± SD

4. Discussion

Although vaccination made diphtheria uncommon disease in some parts around the world, but outbreaks occur all over the world [39-40] *Corynebacterium diphtheria*e PW strain number (8) was used in this experiment for production of diphtheria toxin due to its low infectivity and high capacity for toxin production. This strain was recommended by World Health Organization [10]. Although the classic Linggood medium used in fermentation process contains beef enzymatic hydrolysate which gives high toxin yields [11], this medium has many problems [12-13] and more alarming potential threat comes from Bovine Spongiform Encephalopathy or mad cow disease [27]. Also, one of the most disadvantages of meat extract medium is that the chemical binding between protein molecules from the meat digest and toxoid molecule itself, even the subsequent purification steps cannot eliminate these impurities and purity of toxoid can reach to 60-70% [41-10]. On the other hand, no completely synthetic medium has been proposed to support the growth of *C. diphtheria*e to produce high yield of diphtheria toxin [14-12]. In the present study, we tried to modify the classic Linggood medium by substitution the meet used by beef extract. The obtained results showed that the utilizing of modified semi synthetic Linggood medium for the growth of *C. diphtheria*e gave high yield of diphtheria toxin. Moreover, the SDS result revealed that the semi synthetic Linggood medium produce highly purified toxin (lack of highly undesirable impurities) in comparing with meat-based classic Linggood medium. These results are in accordance with Stainer and Scholte [12] who elucidated that diphtheria toxin could be produced with high potency in submerged culture using a semi synthetic medium. In the same approach, Tchorbanov *et al.* [10] reported that the use of semi synthetic casein-based medium instead of meat extract based broth produced highly purified diphtheria toxoid. Chung *et al.* [42] proved that highly pure diphtheria toxoid was produced from casein –based medium in fermenter. In this study, a five liter capacity fermenter was used for cultivation of the bacteria to produce DT showed the growth of bacteria in term of optical density was 2.1, amount of toxin titer was 60 Lf/ ml and dry cell mass was 1.6 g/l, which indicate for the more growth with high yield of DT. These results are in harmony with Moghadam and Afsharpad [43], who showed that the production of *C. diphtheriae* in fermenter was increased six times than the production through the old static method, and showed that the cultivation of 350 L of *C. diphtheriae* in fermenter technique for 40 hr. gave toxin titer (100-250 Lf/ml) in contrast with the cultivation by the classic method which consumed 168 hr. to produce toxin titer (50-100 Lf/ml). Also, our results in agreement with Tchorbanov *et al.* [10], who illustrated the importance of pH in culture medium during the cultivation for production of DT.

The most commonly methods used for inactivation for vaccines preparation are heat or formalin treatment [16]. Although the formaldehyde is the most common chemical used in vaccine industry [44], but unfortunately it has several disadvantages, like possible irritation, pain and discomfort following vaccination besides, irreversible modifications which could occurred by cross-linking antigens which can damage key antigenic epitopes leading to reduction in immunogenicity or result in unexpected effects [18-45]. Moreover the hazardous reaction between formalin and toxin molecules cannot be abolished [46-17]. Therefore, the need exist for safe and convenient method to inactivate diphtheria toxin. Among the practical applications of radiobiological techniques that may be of considerable interest for public health is the use of ionizing radiation in the preparation of vaccines [21-47]. Radiation could be used to produce toxoids and vaccines; it successfully employed to modify biomolecules, by reducing or abolishing their biological activity without affecting their immunogenic properties [48]. The present results of protein content for irradiated toxoids revealed that the safety effect of radiation with doses 0.5, 2, 5, 10 KGy and standard toxoid. Whereas, by increasing the dose of radiation at doses 15, 25 and 40 KGy the protein content decreased. In this respect of using high doses of irradiation 15, 25 and 40 KGy, Alexander and Hamilton [49] showed that irradiation of protein revealed to damage of amino acids side chains, production of new groups, splitting of peptide bonds and formation of intramolecular and intermolecular cross links. Also, these results are in harmony with Gaber [50] who observed that increasing the gamma radiation dose decreased the molecular weight of the bovine serum albumin due to the effect of gamma radiation on protein which caused disruption of the ordered structure of protein molecules as well as cross-linking, degradation and aggregation of polypeptide chains. The present results of SDS analysis showed no change in the protein bands of gamma radiation and formaldehyde diphtheria toxin, these results are in accordance with Boni-Mitake *et al.* [51] who showed that gamma irradiation has no effect on the molecular weight of crotamine venom. Also, Costa, [52] proved that gamma irradiation has no effect on the molecular weight of *Apis mellifera* venom. In contrast, Caproni *et al.* [48] showed that gamma radiation with dose of 2KGy affected on the molecular weight of bothrops toxin-1 and crude venom *of Bothrops jararacussu*. Also, Gabra *et al.* [53] elucidated that irradiation of *Echis coloratus* venom with doses 2, 5 and 10 KGy caused change in protein bands. Sivananda *et al.* [54] demonstrated that mammalian cell lines like Vero cells can use as alternative to vivo assay for toxicology study of DT because the vitro assay reduce the high costs of vivo assay. The present work showed that gamma irradiated toxoids with different doses (0.5, 2, 5, 10, 15, 25 and 40 KGy) and formaldehyde toxoid

have a non-toxic effect on the Vero cells but the diphtheria toxin caused destruction for Vero cells. These results are in accordance with Abib and Laraba-Djebari [55] who showed that the dose range from 25-80 KGy were able to completely inactivate the partially purified Salmonella enterotoxin as confirmed by CHO cells assays. Also, the present results agree with Domijan *et al.* [56] who revealed that the gamma irradiated mycotoxins with doses 5 and 10 KGy have less toxic effect on HepG2 and PK15 than the non-irradiated toxin.

The liver is a major producer of most serum proteins and regulates their total levels in the blood, so sera ALT and AST levels are known to be good markers for hepatic dysfunction [57]. The present study showed that there was no significant difference in the ALT and AST level between irradiated toxoids at doses 0.5, 2, 5, 10 and 15 KGy and control. These results are in agree with Abdou *et al.* [58] who demonstrated that there is no significant increase of ALT and AST level with gamma irradiated snake venom (*Naja Nigricollis*) at 1.5 KGy dose. Also, Samy *et al.* [59] approved that there is no significant elevation in the activities of ALT and AST level with a single injection of sub lethal dose at 3 KGy. Besides, Gabra, *et al.* [53] revealed that injected mice with different doses of gamma irradiated *Echis coloratus* venom at 2, 5 and 10 KGy showed decrease in the rise of ALT and AST than crude venom. However, the present study revealed that the high doses of radiation (25 and 40 KGy) and formalized toxoid caused elevation of ALT and AST levels. The kidney preserving overall fluid balance, adjusting and filtering minerals from blood and wastes from food, urea and creatinine levels are indicators for kidney activity [60]. The current work showed there was no significant difference in the level of urea and creatinine between all toxoids groups and as compared to control. These results are in agree with Gabra, *et al.* [53] who revealed that there was insignificant change in level of creatinine in groups treated with LD_{50} of irradiated (2 and 5 KGy) of *Echis coloratus* venom.

5. Conclusion:

The present study revealed that the semi synthetic medium produce purified diphtheria toxoid and gamma radiation at doses 0.5, 2, 5, 10 and 15 KGy can be used in the detoxification of DT to produce safe and effective diphtheria toxoid.

6. Conflicts of interest:

There are no conflicts to declare

7. Funding:

There are no funding sources.

8. Acknowledgments

The authors are grateful thanks to the valuable support from Egyptian Atomic Energy Authority and faculty of Science, Ain Shams University, also deep thanks to VACSERA authorities for supporting and facilitating this work.

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