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Radiation-sterilized Carabao Serum as an *In Vitro* Tissue Culture Supplement

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In this study, radiation-sterilized carabao serum (RSCS) was used as a tissue culture supplement for culturing tumor cells *in vitro*. It served as a substitute for expensive commercial cow sera. Microbiological tests showed that sterility of fresh carabao serum can be achieved at 6 kGy. Although some physico-chemical properties (pH, viscosity, UV/VIS absorption spectrum and HPLC profile) indicated a generalized radiolytic phenomenon, its biologic performance as a tissue culture supplement for A549 lung carcinoma cells was still acceptable.

Keywords: radiation processing, radiation sterilization, serum, carabao, tissue culturing, radiolysis

Fetal calf sera (FCS), as well as adult sera from cattle are being marketed as key components in animal tissue cultures. In a typical laboratory research, for example, to sustain a cell culture laboratory for developing monoclonal antibodies from SP2/0 hybridoma cells, a 500-ml bottle of FCS costing P15,000 can be consumed within 2-3 weeks. To produce the desired final product, in this case, purified mAbs for diagnostics, the hybridoma cells need to be continuously cultured and manipulated *in vitro* for several months. Presently, the rapidly growing field of *in vitro* tissue culture technology has already yielded biotechnology products ranging from bioactive designer drugs to artificial organs. Amidst these tremendous advancements in the international community, given that high-level tissue-culturing work have started in the Philippines for more than a decade, we surmise that the unavailability of a self-sustainable biotechnology-based resource may pose as a constraint among local scientists working on small research funds. This article presents our study in search of a local cell culture supplement from the serum of adult Philippine swamp-type buffalo/carabao using radiation-processing technology.

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Materials and Methods

Serum Preparation

Fresh blood was collected from adult male carabaos from an abbatoir in Novaliches, Metro Manila. The collected blood was allowed to coagulate at 4°C overnight and centrifuged at 10,000 rpm for 15 mins at 4°C to separate the serum. Heat-inactivation was performed at 56°C in a thermostatically-controlled water bath shaker (Thermolyne) to remove heat-labile, complement proteins. Serum was later aliquoted into

cryotubes and immediately stored at -80°C. Samples were irradiated at 0°C at the Co-60 pilot-scale irradiation facility of PNRI at graded absorbed doses of 2-12 kGy.

Microbial Analysis

Total plate count per 2 ml of the aliquot was determined using the standard pour plate method after 48 hr-incubation at 37°C in a microbial incubator. Predominant bacterial colonies were isolated after streaking onto a nutrient agar (3 g/l beef extract, 5 g/l peptone and 15 g/l agar) (Difco). The following biochemical tests and staining techniques were employed for culture identification (Collins, Lyne & Grange, 1995).

Morphological and growth assessment of colonies

Pure colonies plated on solid nutrient agar were characterized based on the morphological characteristics of colonies: border shapes, surface texture, pigmentation, opacity, structure, consistency, emulsifiability, odor and elevation. Growth attributes in the liquid media was monitored using the following parameters: surface growth, sediment formation, odor, pigmentation and turbidity.

Staining. Smears of the microorganisms were prepared on slide. The following staining techniques were performed: (a) simple staining with crystal violet, (b) gram-staining with crystal violet with safranin counterstain (c) endospore staining with malachite green with safranin counterstain. The smears were examined under HPO (40x) and oil-immersion objective (100x).

Motility test. Samples of the test microorganisms were suspended in 0.1% peptone solution in a hanging-drop slide. Motility was examined under the oil-immersion objective.

Catalase test. A portion of the colony was blotted on a slide. A drop of hydrogen peroxide was added and formation of bubbles was monitored.

Gelatin-hydrolysis test. The organisms were stab inoculated into 15% gelatin and incubated at room temperature for 14 days. The tube was later placed at 4°C for 1 hour and degree liquefaction of gelatin was noted.

Physico-Biochemical Tests

The following parameters were studied: pH, viscosity, UV/VIS absorbance spectrum and high-pressure liquid chromatography (HPLC) separation. pH was read using Beckman P50. Viscosity was determined in a Brookfield Digital Viscometer Model DV-II using spindle 14 at 60 rpm. Absorption spectrum was read using a Spectronic 21D Milton Roy spectrophotometer. Reversed-Phase HPLC was employed to examine radiolytic products in the serum samples. Five hundred μl of diluted serum (1:10) was injected and adsorbed onto a C-18 column using acetonitrile:water as mobile phase with flow-rate set at 1 ml/min. Absorbing species were detected with a 215 nm-uv detection system. Bovine serum albumin (BSA) (Sigma Chemicals) was used as standard.

Brine Shrimp Cytotoxicity Assay

Desiccated brine shrimp eggs were placed in seawater solution inside an improvised brine shrimp hatching tank. The tank consists of two chambers: one is a dark chamber where the eggs are placed and the other is the nauplii-collecting chamber. After 48 hours, 100 ml of salt solution containing the nauplii was dispensed to individual wells of a 96-well microtiter plate. Approximately 10-15 shrimps were placed in each well. One-hundred ml dilutions (10, 15 and 20 v/v) in triplicates of irradiated serum were added. The set-up was incubated in the dark for 48 hours. Percentage of dead shrimps was counted under the microscope. Non-irradiated sera at various dilutions served as negative control.

Tumor Cell Assay

A549 lung adenocarcinoma cell line from the American Tissue Type Culture Collection (Rockville, MD) was maintained in RPMI 1640 medium supplemented with 10% FCS (Gibco BRL, MD) at 37°C in a 5% CO_2 environment. The cells at logarithmic phase, approximately 10^6 cells per well, were seeded at various RSCS concentrations (10, 15, 20, 25 and 30% in RPMI 1640) at six replicates per treatment. Viability of the cells was quantified using a start-point MTT assay (Hansen *et al*., 1989). Briefly, after the incubation period of 4 days, 25 μl of 5 mg/ml MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] (Sigma) was added to each well. The plates were incubated for 2 hours in standard tissue culture conditions. Afterwhich, 100 μl of lysis buffer [20 g sodium dodecyl sulfate (Sigma) in 100 ml 0.5x dimethylformamide (Sigma), pH 4.7] was added to each well. Absorbance was measured in dual wavelengths 450:630 nm in a microplate reader (Molecular Devices, CA). Serum-free and 10% FBS-fortified RPMI 1640 served as controls.

RESULTS

Effects of irradiation of serum microflora

Increasing the absorbed radiation dose led to a logarithmic decrease in total microbial load (**Figure 1**). The most radio-resistant species apparently had a threshold at 5 kGy and beyond that sterility of the

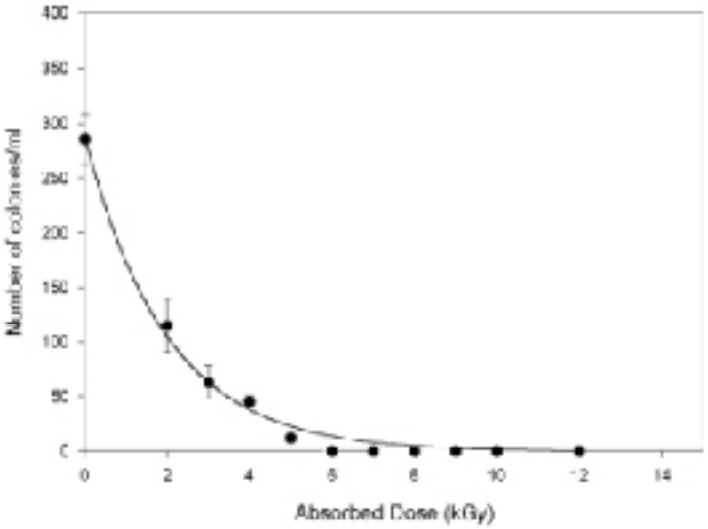

Figure 1. Total plate count of microbial colonies in the carabao serum as a function of radiation dose.

product was achieved. Therefore, it can be deduced that sterilization dose of 6 kGy can be recommended for liquid-state irradiation of future carabao sera under cold condition.

Isolates of the predominant colonies were identified based on the morphological and biochemical criteria (Holt *et al*., 1994). While all the colonies actively populated the raw sera, microbial flora-transitions became evident after irradiation. With the absorbed dose of 2 kGy, *Staphylococci* colonies disappeared. At 3 kGy, inactivation of *Eschericia* and *Streptococci* were evident. Between 4-5 kGy, only *Bacillus* colonies remained. No bacterial colonies survived at higher doses. These microbes represent the contamination load accrued during the serum handling, from blood collection in the abbatoir, serum separation and dispensing at the laboratory prior to radiation-sterilization. Although added precautions were taken such as, the implementation of cold-chain and hygienic handling techniques, the researchers are still beset with initial high microbial load. Notably, colonies of *Baccilus sp*. were the most tenacious, that surviving even after 5 kGy radiation dose. Its radio-resistance was due to their ability to form endospores that are adaptive to extremely harsh conditions (Holt *et al*., 1994).

Radiolytic changes in serum physico-biochemical properties

Viscosity. Viscosity was markedly affected at 6 kGy with a mean 11.7 cPs (non-irradiated) down to 3.9 (6 kGy-irradiated) (p<0.01). Viscosity or the measure of internal friction of a fluid is correlated with the polymeric nature of the serum. The marked decrease in serum viscosity suggests depolymerization (**Figure 2**).

pH. Significant decrease in pH from 8.4 to 8.1 (p<0.01) upon irradiation at the sterilization dose of 6 kGy was observed (**Figure 3**).

Absorption Spectroscopy. No gross change in color and turbidity was noted. The absorption spectrum from 150 to 600 nm revealed the following changes: (a) a hypsochromic shift to a shorter wavelength from 340 to 320 nm, (b) peak depression at 500 nm and (c) disappearance of a peak at 540 nm. The trends were suggestive of radiolytic-degradative species (**Figure 4**).

HPLC Profile. Liquid chromatograms of non-irradiated and 6 kGy-irradiated sera are shown in **Figure 5**. Chromatogram of raw serum consisted of two major peaks with retention time of 5.4 and 6.4 mins. One of these peaks was identified as albumin by a known standard. HPLC analysis of the 6 kGy-irradiated lot showed two major degradative events: (1) shoulder peak that originated from albumin and (2) a small peak at 9.9 mins.

Radiotoxicity of irradiated sera

No radiomimetic products were detected. Shrimps survived up to 15% (v/v) of sera. At higher concentrations, the solution became too viscous for the nauplii.

Cell culture performance

The culturing performance of RSCS at different supplementation levels was compared with 10% FCS as gold standard based on the metabolism of formazan by viable A549 cells (**Figure 6**). ANOVA results indicate that the growth of A549 is a function of RSCS serum supplementation (p<0.05) although 10% FCS was able

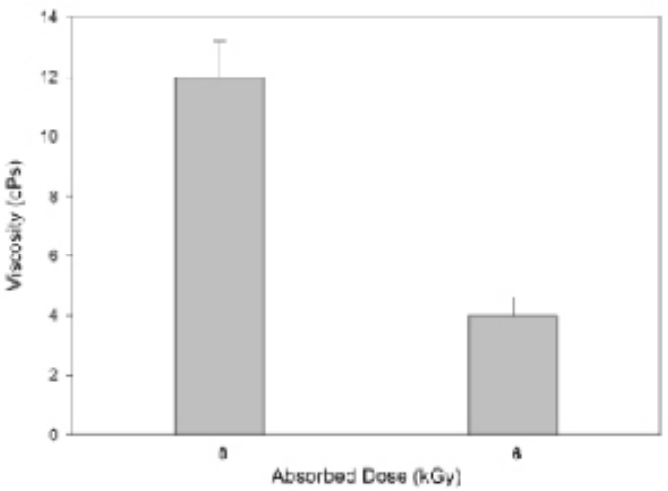

Figure 2. Effect of 6 kGy absorbed dose of gamma radiation on viscosity of carabao serum.

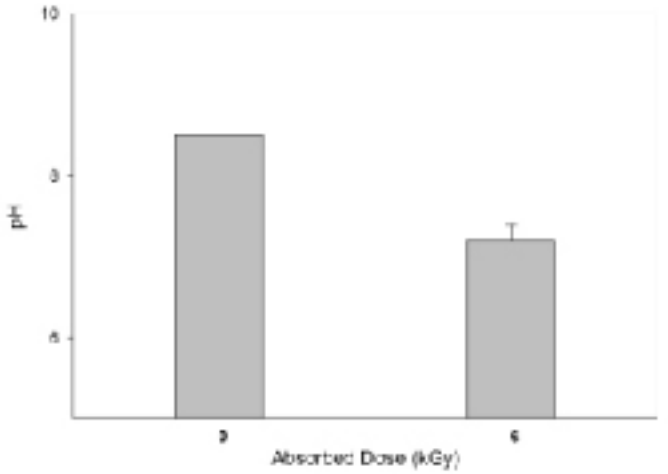

Figure 3. Effect of 6 kGy absorbed dose of gamma radiation on pH of carabao serum.

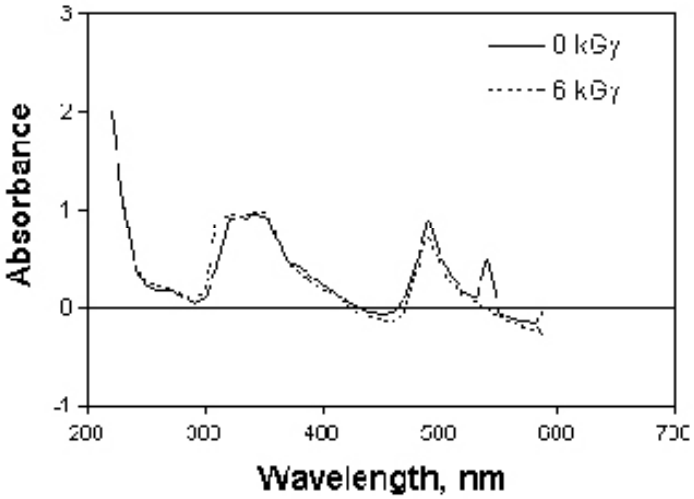

Figure 4. UV-visible spectra of (a) non-radiated and (b) 6 kGy gamma-radiated carabao serum.

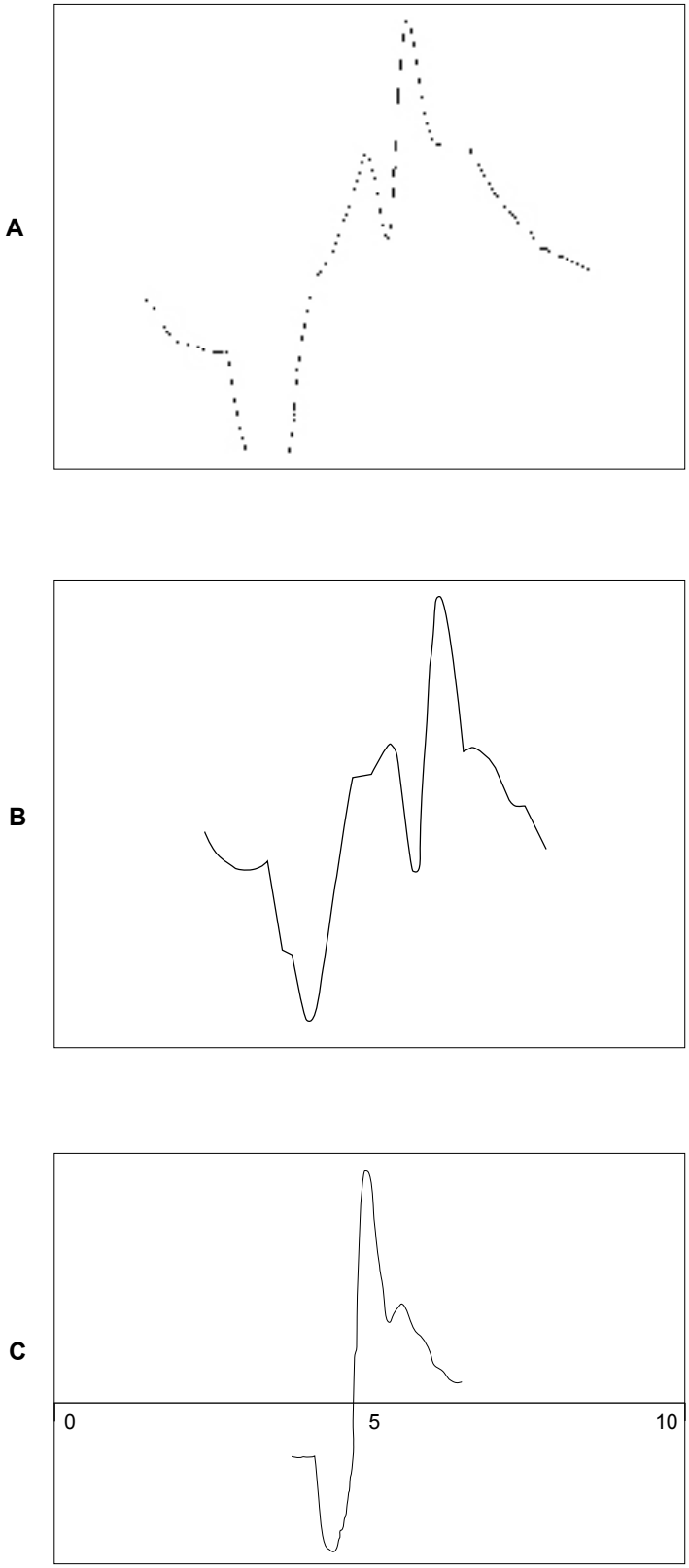

Figure 5. HPLC profiles of (a) non-radiated and (b) 6 kGy gamma-radiated carabao serum. (c) commercial bovine serum albumin (Sigma Chemicals) served as reference for comparison.

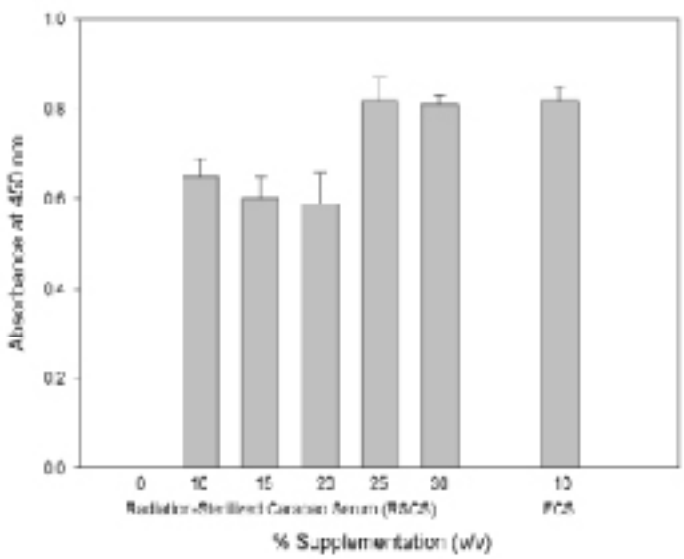

Figure 6. MTT-DMF absorbance of A549 cells after co-culturing with various concentrations of RSCS and 10% FCS.

to give 1.3-fold higher growth potentials than RSCS at the same concentration.

Discussion

In a commercial setting, animal blood for mass-production of research sera is normally obtained through cardiac puncture. The blood is allowed to coagulate and the serum is pooled and immediately frozen under sterile conditions. Prior to bottling, the approved raw serum is allowed to pass through 0.1-mm filters to achieve an Industry Standard Sterility Assurance (ISSA) of 10^{-6}. All glass containers are heat-sterilized in dry depyrogenation conditions while plastic containers are γ-irradiated. To achieve utmost sterility, all procedures are performed in positive-pressured, HEPA-filter rooms (Sigma, 2001).

While the complexity and capital-intensiveness of these procedures have a large impact on the market price of research sera, the advantage of γ-radiation is underscored. Radiation-processing has a long history in sterilization of medical products and which has an equivalent sterility requirement of SAL 10^{-6} (IAEA, 1973). From a manufacturer's perspective, this method has an added practical advantage, that is, the raw sera can be processed under ambient conditions and the product can be sterilized at the desired temperature in the final packaged form.

In the raw serum, two contaminating bacterial genera have been identified that could possibly pose future problems in radiation-sterilization of our carabao sera. One is *Bacillus sp.*, with a threshold dose of 5 kGy, a radio-resistant endospore-former. Thriving at 3 kGy, the other group is *Streptococci sp.* that has an endogenously efficient adaptive DNA repair activity (Christensen, 1970). Emphasis is also drawn to the presence of *Eschericia sp*. Although it is radiosensitive, this group is indicative of sample exposure to fecal contamination (Thatcher, 1970). Coliforms are likely to be among the major contaminants unless current hygienic conditions in the abattoirs are improved.

It should be noted that initial heavy bioburden of the carabao sera is unwarranted. Microorganisms may bring about degenerative changes in the chemical composition of the serum and their metabolites may manifest undefined cytopathic alterations. Excess bioburden can also lead to gross failure of the irradiation conditions requiring higher levels of exposures. It has been reported that increasing the radiation dose exposure for sera in liquid-state to more than 15 kGy to even destroy viral contaminants will not seriously generate radiomimetic or cytotoxic components (Deocaris *et al*., 1999). However, these extra "kGy*s*" would pose as additional costs and could seriously compromise the quality of the serum.

Serum is an extremely complex mixture of biomolecules such as proteins (essential and non-essential), hormones, nutrients and metabolites, minerals and salts and some inhibitors and protective agents (Freshney, 1994). Although the identity this plethora of biomolecules remains obscure, a number of agents have been isolated and their functions delineated. While it is impossible to determine the response of each component to radiation, we have gauged through physico-biochemical parameters some effects of radiation-sterilization on serum component.

A decrease in pH is likely attributed to the release of free amino acids in the solvent. Loss of viscosity may be attributed to the degradation of large polymeric molecules, e.g. residual clotting components and albumin. In fact, based on the HPLC profile, one of the damaged components identified was albumin. These biochemical changes in the proteins are the following: (1) denaturation of protein folding by disruption of intra- and inter-molecular H-bonding, (2) chain-scission of the peptide backbone, (3) deamination and decarboxylation of amino acids, (4) opening of aromatic groups and (5) oxidation of sulfide bonds. In addition, higher levels of organization of protein are more sensitive and this is enough to affect bioactivities. However, the dose range of 6 kGy for serum treatment is still low. Hence, it is expected that effect on enzymes is minimal based on the experience of several groups working in field of food irradiation within the 10 kGy ceiling dose (ICGFI, 1992). Other components such as vitamins A and E and triglycerides are resistant to doses below 50 kGy (Dole, 1973). Radiolytic damage to the biological components, nevertheless, is the pay-off in achieving sterility. Additional processing conditions, such as irradiation at low temperature, aside from controlling proliferation of microbial contaminants, can minimize free-radical damage and interactions of excited and ionized serum components (IAEA, 1973). Stringent good manufacturing processes during pre-serum processing can minimize microbial contamination and therefore allow one to sterilize the product at a lower dose while minimizing formation of radiolytic products.

The applicability of a radiation-processed serum product lies in the ability to support growth of the cells *in vitro*. A549 is a suitable model system for the study because it is highly neoplastic, and hence, has a high proliferative capacity (Giard *et al.*, 1972). The results of the MTT assay after 4 days incubation show that RSCS-supplemented media are able to support growth of the A549 lung tumor cells, although proliferation potential is 1.3-fold lower compared to commercial FCS. To achieve an equivalent growth-promoting activity, it is estimated that RSCS should be given at 20-25% supplementation. Absence of toxic response to brine shrimp nauplus test is consistent with the response of the tumor cells. Considering the potential lower production cost of RSCS, it would still be practical in the long run even if one doubles the amount of RSCS in the culture medium as compared with standard 10% supplementation with commercial sera.

Among the physical changes observed after radiation processing, viscosity appears to be the most affected. This property becomes important to cultured cells in preventing mechanical damage during agitation. This significant decrease in viscosity may be circumvented by addition of polymers, such as carboxymethyl cellulose or polyvinyl pyrrolidone (Birch and Pirt, 1971) during RSCS-supplementation.

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