

Research

Potential Therapeutic Applications of Extract Made from Electroporated *Xenopus laevis* frog Oocytes in Murine Models of Melanoma, Traumatic Brain Injury and Experimental Skin Wrinkling

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Abstract

We previously demonstrated that co-electroporation of *Xenopus laevis* frog oocytes with normal cells and cancerous cell lines induces the expression of pluripotency markers. Here, we investigated the effects of a combination of intra- and extra-oocyte liquid phases of electroporated *X. laevis* frog oocytes (Bioquantine™) on experimental murine models of melanoma, traumatic brain injury (TBI), and skin wrinkling (induced by squalene-monohydroperoxide). Experimental animals received intraperitoneal injections of 100 µl of 5 mg/ml Bioquantine extract daily. In the melanoma model experiments, mice that received Bioquantine displayed rapid tumor shrinkage. By day 45, the tumors had shrunk by almost 99%, and the animals recovered with no gross signs of re-occurrence during 3 months of post-therapeutic observation. In the TBI experiments, mice that received Bioquantine for up to 20 days showed brain recovery at a gross morphological level with relief of brain edema. These treated mice also showed substantial improvements in spatial memory in the Morris Water Navigation Test. In the squalene-monohydroperoxide-induced wrinkling model, mice that received Bioquantine extract daily for 25-45 days exhibited skin regeneration. These experimental murine model studies revealed Bioquantine extract has potential as a novel treatment for a wide range of conditions including melanoma, TBI, and skin wrinkling.

Keywords: *Xenopus laevis* Oocytes; Melanoma; Traumatic Brain Injury; Skin Wrinkling

Introduction

There is currently a major unmet medical need for novel pharmacological agents that have the ability to alter the biological states that precede the pathophysiological abnormalities of dysfunctional tissue and organ systems. Extracts prepared from *Xenopus laevis* oocytes have the capacity to reprogram somatic mammalian cells into a pluripotent state.¹ Major events in the reprogramming of somatic cells with *X. laevis* extracts include remodeling of the structure of the nucleus, reprogramming of transcription, DNA replication, epigenetic modification of DNA and histones, and production of embryonic proteins [1].

The most common method of preparing *X. laevis* oocyte extracts is by centrifugation at a speed that crushes the cells.¹ In previous studies, we explored electroporation as a means of extracting the components of *X. laevis* oocytes with reprogramming potential.

We demonstrated that co-electroporation of *X. laevis* frog oocytes with normal cells and cancerous cell lines induces expression of pluripotency markers [2].

Preliminary mass-spectrophotometry results identified 93 proteins based on *Xenopus laevis* known protein structure database including vitellogenin-A2, cofilin -1-A, hypothetical protein LOC100101274, and muscle pyruvate kinase. We also studied profiles of 15 miRNAs including: hsa-miR-17-5p, 18a, 92a, 19b-1, 20a, 106b, 302a, 302b, 302c, 302d, 367, 372, 373, mmu-mR92a, and 93, all of which are probably enrolled in the combinatorial somatic cell reprogramming dynamics [14,15]. Preliminary results of protein/microRNA profiling of extracts isolated from electroporated *Xenopus* oocytes may help to further understand the complex mechanisms underlying in-vivo effects and future development for therapeutic applications in human disease modification.

Clinical interest in cellular reprogramming has largely focused on the in vitro production of stem cells for use as therapeutic grafts or implants [3]. Here, we present the results from investigation of direct therapeutic effects of *X. laevis* oocyte extracts prepared using electroporation administered to mice in experimentally induced pathologies including melanoma, traumatic brain injury (TBI), and skin wrinkling induced by squalene-monohydroperoxide (Sq-OOH).

Methods
Ethical Conduct

Bioquark Inc is committed to conducting quality animal research in

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an ethical and responsible manner to further science and to improve the health of society. Bioquark's Institutional Animal Care and Use Committee (IACUC) is responsible for reviewing all protocols involving live vertebrate animals, ensuring compliance with federal regulations, inspecting animal facilities and laboratories and overseeing training and educational programs. Bioquark Inc. is committed to ensuring that the approval of all animal research takes into consideration the possible *replacement* of animals, the *refinement* of studies to alleviate or minimize potential pain and maximize the comfort and welfare of the animals, and the possible *reduction* in the number of animals necessary to obtain valid scientific data. All research personnel completed the required IACUC training and enrolled in the Laboratory Animal Exposure Risk Assessment Program. Procedures involved prolonged manipulation and were invasive. Protocols involved surgical and other stimuli inducing pain or distress, but all pain or distress was mitigated with appropriate anesthetics or analgesics. The procedures were initially approved by staff veterinarian and one additional member of the IACUC. All protocols were reviewed thoroughly by the IACUC prior to commencement of the project.

Animals

Egg-bearing *X. laevis* frogs (Xenopus Express, Inc, Brooksville, FL) were maintained in carbon-filtered water containing 13.3 g/gallon sodium monochloride at ~18°C and 12h / 12h light/dark cycle and fed with brittle (NASCO cat. # SA02764LM, Madison, WI). Water was replaced daily.

Immunocompetent mice aged 3-4 weeks were purchased from Petco Warehouse, Wesley Chapel, FL). Males were separated from females and distributed into cages with 5 mice per cage. Each experimental and control group consisted of 10 mice. Animals were kept on a normal day-night cycle (LD 12h:12 h) and fed with Kaytee Forti-Diet food. Both experimental and control mice were used in these studies starting at age 8 weeks.

Preparation of Bioquantine™ Extracts

Purified sterile and pathogen free Bioquantine extracts were isolated from electroporated *Xenopus Laevis* frog oocytes using proprietary protocol for oocyte activation.

Preparation and Maintenance of *X. laevis* Oocytes: Frogs were anesthetized in 2L of 0.2 % tricaine solution (Sigma cat. # A5040) for 20 minutes and, then were placed on cold dissecting pan. A small incision (0.5 cm) was made through the skin and muscle layers. Ovaries were surgically removed and placed into oocyte washing (OW) solution (82.5mM NaCl) (Sigma cat. #S3014), 5.0 mM HEPES (Sigma cat. #H4034), 2.5 mM KCl (Sigma cat. #P5405), 1 mM MgCl₂ (Sigma cat. #M0250), 1.0 mM Na₂HPO₄ (Sigma cat. #S3264), supplemented with 0.5 % pen/strep and titrated to pH 7.4. Bags with oocytes were disrupted with fine forceps, rinsed 4 times in OW and rest of follicular layers were digested at room temperature in 0.2 % collagenase type II solution (Worthington Biochemical Corporation cat # LS004176, Lakewood, NJ). Defolliculated oocytes were rinsed in the OW solution and placed for overnight incubation in holding buffer (HB) containing 5 mM NaCl, 5.0 mM HEPES, 2.5 mM KCl, 1 mM MgCl₂, 1.0 mM Na₂HPO₄, 0.5 % pen/strep, 1.0

mM CaCl₂ (Sigma cat. #223506), 2.5 mM pyruvate, and 5 % heat-inactivated horse serum (Sigma cat. # H1138) with pH 7.4. Healthy oocytes were collected next day into sterile 6-well clusters (Costar cat. # 3516) filled with HB solution and then incubated at 17 °C for 24 hours before being placed into electroporation flasks.

Electroporation and Activation of *X. laevis* oocytes: Approximately 1,000 oocytes were transferred to 2-electrode T25 cell culture flasks containing OW and electroporated at 750 volts (125 v/cm). After electroporation, 100 µl of energy mix (Creatine Phosphate, ATP and MgCl₂) was added to the oocytes, which were then incubated 3h at 17 °C for 3 hours for recovery and active semiochemical emission.

Preparation of Intra- and Extra-oocyte Bioquantine Extracts: Oocytes were centrifuged at 52 x g for 7 minutes at 4°C. The supernatant, containing extra-oocyte electroporate, was removed and filtered through 0.2 µm filter units (Nalgene cat. #121-0020, Rochester, N.Y.). For preparation of intra-oocyte extracts, the pellet obtained from centrifugation containing activated oocytes was centrifuged at 10,000 rpm (17,000 g) for 15 minutes at 16°C to crush the eggs. The layer containing intra-oocyte extract was carefully removed. Extra- and intra-oocyte extracts were combined in equal volumes into the final composite extract Bioquantine and then protected from RNase/protease damage by adding RNase and protease inhibitors at a final concentration of 1 u/ µl (Applied Biosystems cat. # AM2694 and Sigma cat. # P8340). Bioquantine was incubated for 20 minutes, then purified by passage through cooled 0.2 µm sterile filter units and kept at 3°C.

Determination of Bioquantine Concentration: Aliquots of 150 ml of Bioquantine were distributed into pre-weighted 15 ml lyophilization vials using Auto Internal Calibration Analytical Balance (J&G SCIENTIFIC) and were lyophilized overnight (VIRTIS model 6KBTES-55). Each vial was re-weighted after lyophilization, and the weight of each empty vial before lyophilization was subtracted from the weight of same vial containing lyophilized Bioquantine powder. Bioquantine concentration and mean and standard deviation was calculated using arithmetic standard deviation calculation software (<http://easycalculation.com/statistics/standard-deviation.php>).

Mycoplasma and Bacterial tests for Bioquantine Extracts: Bacterial tests were conducted using Gram Staining Kit (Fluka cat. # 77730). Mycoplasma contamination testing was conducted using PCR-based Universal Mycoplasma Detection Kit (ATCC cat. # 30-1012K)

Bioquantine Administration

In the experimental melanoma, TBI, and skin wrinkling experiments, mice received daily intraperitoneal injection of 100 µl of 5mg/ml extract of Bioquantine for up to 45 days while negative control mice received sterile deionized water.

Murine Foot Pad Melanoma Experiments

B16 melanoma cells were purchased from American Type Culture Collection (ATCC, cat. # CRL-6323). The vials were thawed, and the cells were washed in phosphate-buffered saline and then grown at

37 °C and 5 % CO₂ in DMEM/10% (v/v) fetal calf serum/2 mM L-glutamine/100 U/ml penicillin/100 µg/ml streptomycin. When the culture reached confluency, the cells were detached by trypsinization, washed, counted, and diluted in PBS solution to concentration of 10⁶ cells/ml. A total of 100 µl of solution containing around 10⁵ melanoma cells were subcutaneously injected into left or right food pads of mice. Palpable primary tumors were first detected between day 12 and 14 day after injection. Therapeutic injections of Bioquantine were started on day 24 after mice were injected with B16 melanoma cells. 3-Dimensional measurements of tumors were conducted using a digital 6" Inside Groove Vernier Caliper.

Expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) was assessed in formalin-fixed paraffin-embedded sections of mouse food pad melanoma tissue by immunohistochemistry (IHC). Primary antibodies consisted of an anti-iNOS rabbit monoclonal antibody (1:50) (Labvision, CA, USA) and an anti-COX-2 mouse monoclonal antibody (1:50) (Transduction Laboratories, Lexington, KY). Tissue sections were de-paraffinized and rehydrated, then placed in a 0.01 M citrate buffer, pH 6, and microwaved intermittently for a total of 20 minutes. After cooling, the slides were placed in 3% H₂O₂ in H₂O for 30 minutes. After 30 minutes of blocking in 1% BSA, the primary antibody was applied overnight at +8°C, followed by 30 minutes' incubation with biotinylated secondary antibody, and an avidin-biotin-peroxidase complex kit (Vectastain, Vector Laboratories) was used for antigen detection. The immunostain was developed with the chromogen 3-amino-9-ethylcarbazole for 6 min. Hematoxylin was applied as a counter stain. Colon carcinomacells (mouse CT-26 colon carcinoma cells induced by methylcholentherene injection) with known COX-2 and iNOS expression were used as a positive control for immunostaining. Normal foot pad tissue samples from control animals were used as negative controls. No positive controls used in IHC studies. Immunostained tissue sections were scored separately for two variables: the proportion of iNOS and COX-2 positive cells and for the overall intensity of immunoreactivity of the positive cells. Briefly, scoring for number of positive cells was defined as follows: "0", <5% positive cells; "1", 5–25% positive cells; "2", 25–75% positive cells; "3", greater than 75% positive cells. Intensity scoring was defined as follows: "0", no staining; "1", weak staining; "2", moderate staining; and "3", intense staining.

TBI Experiments

The mice were briefly anesthetized with ether within an excicator and then placed on a Weight Drop Mechanism (WDM) platform. Animals were immobilized using magnetic clips. Standardized 200g weight drop released from 4 cm height induced a focal blunt injury over an intact mouse skull. The resulting impact produced a closed head injury with profound neuro-inflammatory response within the intrathecal compartment that included including bleeding and brain swelling.

Memory testing was conducted using the Morris Water Navigation test.⁴ Prior to induction of TBI, mice were trained to find an escape platform hidden 1.5 cm below surface of water; the location can normally be identified by using spatial memory. Trajectories of

swimming animals were monitored by video equipment mounted above the center of the 180 cm Ø circular pool.

Immunohistochemical staining of β -amyloid protein deposits in the brains of TBI and control mice was conducted using chromogen 3,3' Diaminobenzidine (DAB). We used clone: 6F/3D, Mouse anti-human beta-amyloid antibody (Novocastra Labs. Cat. #NCL-B-Amyloid) as a primary antibody (1:100 using IHC-Tek™ Antibody Diluent [Cat. # IW-1000 or IW-1001]) to reduce background and unspecific staining. Incubation time was 60 minutes at room temperature. Antigen retrieval was conducted using DAB Chromogen Substrate with incubation time for 1-3 minutes at room temperature. Counterstaining was conducted using Gill's Hematoxylin or Mayer's Hematoxylin for 30 seconds at room temperature.

Skin Wrinkling Experiments

Squalene-monohydroperoxide (Sq-OOH) was synthesized from squalene (sigma cat. # S3626) using a slightly modified version of the experimental protocol described by Chiba et al. [5]. Briefly, 10 ml of squalene was placed in 50 ml beaker and irradiated for 2 hours inside a biosafety cabinet using a Dermalight 80 UV Phototherapy UVB 311 phototherapy system (Munich, Germany) with spectrum 340–440 nm positioned at a distance of 30 cm. UVB irradiation was carried out using a PLS9w/01 (DRH060) UVB light source (Philips, Aachen, Germany). Approximately 8 cm² of mouse right lateral flank skin was depilated using HibrosDepilSport Cream Hair Remover™. Exposed sterile skin received daily topical application of Sq-OOH for up to 3 weeks using a Sterile Master Amp™ Buccal Swab Brush (Epicentre Biotechnologies cat. # MB100SP). Master Amp™ brushes soaked in Sq-OOH. On day 7, changes in the skin were photographed using Nikon Coolpix 14.0 megapixel digital camera. Animals in a negative control group was maintained on the standard diet in stress free environment.

Results

Pathogen Testing

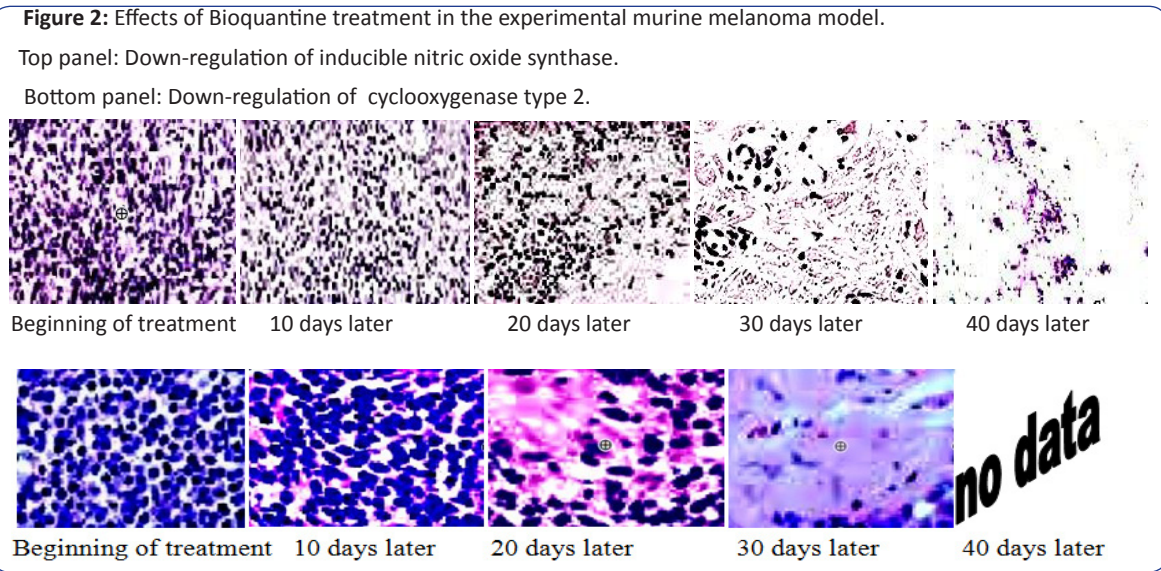
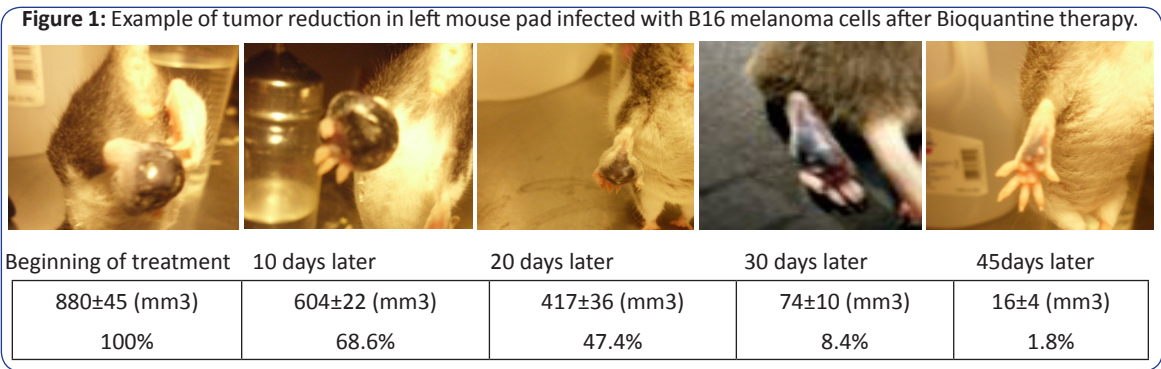
The bacterial and mycoplasma tests of Bioquantine revealed no evidence of these contaminants. Mice receiving intraperitoneal injections of 100 µl of 5 mg/ml Bioquantine daily for up to 12 months did not develop any infectious (e.g., abscess) or inflammatory reactions at the injection site, and did not display any negative behavioral activities or responses.

Murine Foot Pad Melanoma Experiments

Mice that received intraperitoneal injections of 100 µl of 5 mg/ml Bioquantine daily for up to 45 days displayed rapid tumor shrinkage. By day 10, tumors had shrunk by almost one-third. By day 45, the tumors had shrunk by almost 99%, and animals recovered afterwards with no gross signs of re-occurrence during 3 months of post-therapeutic observation (Figure 1). Foot pad tissue from mice that received Bioquantine had substantial down-regulation of iNOS and COX-2 (Figure 2).

Murine TBI Experiments

Animals that received Bioquantine via intraperitoneal injections of 100 µl of 5 mg/ml given daily for up to 20 days showed brain



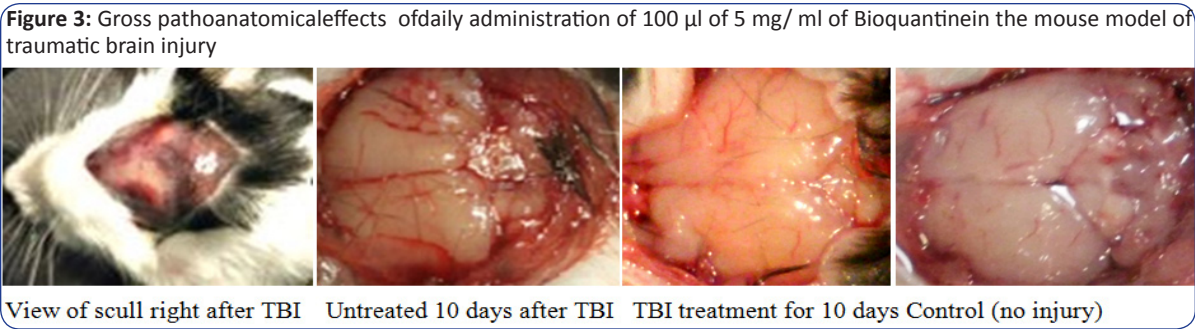
recovery at a gross morphological level with relief of brain edema (Figure 3). Experimental mice that received Bioquantine also showed significant improvements in spatial memory as measured by the ability of mice to find the escape platform in the Morris Water Navigation Test (Figure 4). The TBI mice that received Bioquantine (n=10) were able to find the escape platform 3.8 times more quickly than did TBI mice (n=10) that did not receive Bioquantine. Time to fine the escape platform was similar for TBI mice that received Bioquantine and for normal healthy mice. Statistical comparisons were not performed. Treatment of the TBI mice with Bioquantine was associated with a reduction in expression of β -amyloidprotein in the hippocampus (data not shown).

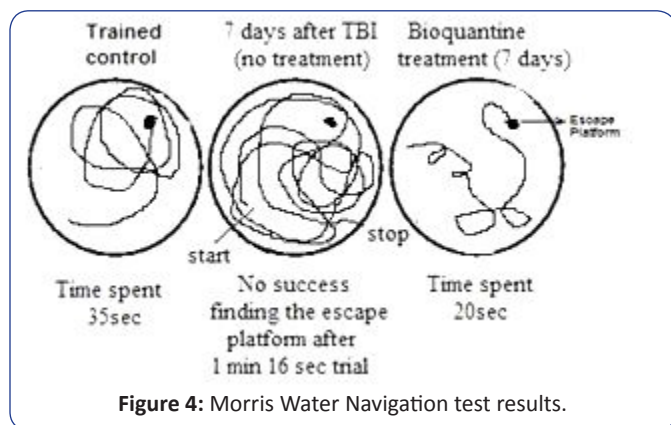
Sq-OOH-induced Wrinkling Experiments

In the Sq-OOH-induced wrinkling model, mice that received Bioquantine extract injected 5mg/ml/day for 25-45 days exhibited skin regeneration. Mice not treated with Bioquantine extract animal did not show the dramatic smoothing of the skin comparable with treated mice (Figure 5).

Discussion

Our previous work with normal and cancer cell lines demonstrated that Bioquantine has cellular reprogramming properties *invitro* [2]. The present studies provide the first evidence of beneficial effects of Bioquantine *invivo* experimental models of cancer, CNS injury, and aging.





ming properties for potential therapeutic applications in oncology, neurodegenerative diseases and CNS injuries [10-14]. We have extended our characterization of Bioquantine, which contains strong tissue self-reprogramming potential preserved in amphibians through millions of evolution [2], to *in vivo* disease models. Although these experiments were preliminary and require research, the data show anti-cancer activity in a melanoma model and regenerative properties in experimental TBI and skin aging. We suggest that these varied effects may be linked to the reprogramming properties of Bioquantine.

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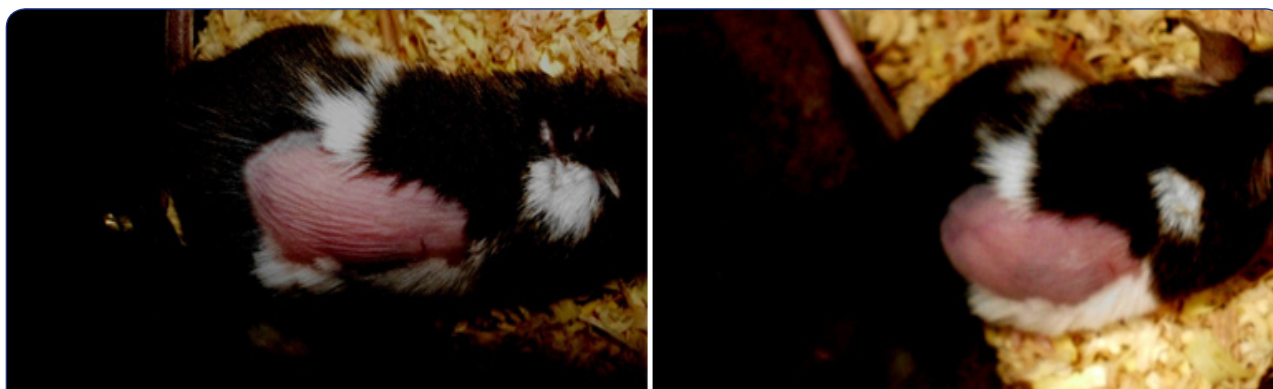


Figure 5: Therapeutic effect of Bioquantine treatment in mice with squalene-induced skin wrinkling model.

Our studies observed that Bioquantine had strong anti-melanoma activity with extensive and rapid tumor shrinkage in a murine experimental melanoma model. This finding was supported by immunohistochemistry results showing that COX-2 and iNOS, two important cancer prognostic markers [6-8], were substantially down-regulated in the affected foot pad tissue. The rate at which these tumors shrank, with almost the entire tumor gone by 45 days of treatment, is of note. The most active treatments currently available for advanced melanoma, immunotherapeutic agents, are associated with a considerably longer time to response (12 weeks)[9].

The TBI study findings indicate that Bioquantine crossed the blood-brain barrier, reduced edema, and improved brain morphology in mice in an experimental model of TBI. Furthermore, Bioquantine therapy helped mice recover spatial memory as shown in their performance in the Morris Water Navigation. This study also showed that Bioquantine therapy may restore a normal level of beta-amyloid protein following brain injury.

The experimental skin wrinkling studies provide evidence of therapeutic dermatologic and regenerative effects of Bioquantine. Treatment with Bioquantine extracts promoted skin regeneration and eliminated squalene-monohydroperoxide (Sq-OOH) induced wrinkling formations.

There is growing interest in compounds with cellular reprogram-

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