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Methodology article

CELL PROLIFERATION ASSAY – METHOD OPTIMISATION FOR *IN VIVO* LABELING OF DNA IN THE RAT FORESTOMACH

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The study of cell proliferation is a useful tool in the fields of toxicology, pathophysiology and pharmacology. Cell proliferation and its degree can be evaluated using 5-bromo-2'-deoxyuridine which is incorporated into the newly synthesized DNA. The aim of this study was the optimization of subcutaneous application of 5-bromo-2'-deoxyuridine implantation for continuous and persistent marking of proliferating cells in the rat forestomach. 3-*tert*-Butyl-4-hydroxyanisole was used as the agent that ensures cell proliferation. In order to determine the optimal dose for proliferating cells labeling, 5-bromo-2'-deoxyuridine doses of 50 mg, 100 mg, 200 mg or 350 mg were implemented 2 days prior to sacrifice by flat-faced cylindrical matrices. Immunohistochemical analysis using 5-bromo-2'-deoxyuridine *in situ* detection kit was performed for the detection of 5-bromo-2'-deoxyuridine labeled cells. The results showed that for adult rats, the optimum 5-bromo-2'-deoxyuridine dose is 200 mg per animal for subcutaneous application. The here described manner of 5-bromo-2'-deoxyuridine *in vivo* labeling provides a simple, efficient, and reliable method for cell labeling, and at the same minimizes stress to animals.

Key words: 5-bromo-2'-deoxyuridine, 3-*tert*-Butyl-4-hydroxyanisole, BrdU *in vivo* labeling; cell proliferation; rat forestomach

INTRODUCTION

The rat stomach is morphologically and physiologically different from the human and the stomach of other laboratory animals. Its proximal nonglandular part, i.e. the forestomach, constitutes approximately 60% of this organ and it consists of a stratified squamous epithelium which is constantly renewing [1]. Therefore, the balance between

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cell proliferation and cell loss by apoptosis is necessary for the normal functioning of the forestomach.

The main function of the forestomach is to serve as a food reservoir leading to long-term exposure to xenobiotics, which makes it a good target tissue in cell proliferation studies [2]. Numerous xenobiotics lead to disturbed tissue homeostasis, which may result in cell loss, consequent tissue atrophy and failure, or excessive cell proliferation and eventually carcinogenesis, which makes cell proliferation studies a very important and useful tool.

The forestomach is sensitive to tissue damage by non-genotoxic compounds like the food additive 3-*tert*-Butyl-4-hydroxyanisole (BHA). BHA is commonly used as an antioxidant food additive in fats and oils (E321). There is evidence that prolonged exposure to this agent induces hyperplasia that eventually results in carcinomas in the rat forestomach [3,4]. On basis of such data the International Agency for Research on Cancer (IARC) has classified BHA as a B2 carcinogen [5].

The study of cell proliferation is a useful tool in the fields of toxicology, pathophysiology and pharmacology [6,7]. Cell proliferation and its degree can be evaluated using 5-bromo-2'-deoxyuridine (BrdU) which is incorporated into the newly synthesized DNA, during the S phase of the cell cycle [8]. BrdU is a thymine analogue which pairs with purine bases with different affinity depending on its isoform, i.e. in the keto form it pairs with adenine, whereas in the enol form it pairs with guanine [9]. This method allows the quantification of newly generated cells and therefore enabled the study of the effects of xenobiotics on cell proliferation, death and migration behavior in a quantitative manner [10]. Pharmacokinetics studies showed that the most commonly used doses of BrdU (50-100 mg/kg) and routes of administration such as intravenous and intraperitoneal (i.v. and i.p., respectively) are suitable for use in pulse-label experiments. BrdU is quickly degraded *in vivo*, and its half-life is approximately 60 min both in the blood serum and in various tissues [11,12]. For continuous-label studies a prolonged and continuous exposure to the labeling agent must be achieved.

Although several administration routes of BrdU have already been described in order to mark proliferating cells, accurate monitoring of proliferation in tissues that need more time for renewal requires further optimization. Furthermore, the effects of subacute or chronic exposure to proliferating agents could be observed more precisely with continuous-label methods. The aim of this study was to optimize a subcutaneous BrdU implantation for continuous and persistent marking of proliferating cells. As the agent that ensures cell proliferation we used 3-*tert*-Butyl-4-hydroxyanisole (BHA) [3].

MATERIALS AND METHODS

Animals

All procedures on animals were approved by the Ethical Committee for the Use of Laboratory Animals, University of Belgrade, Vinča Institute of Nuclear Sciences

(Number of Ethical Committee approval DD1/15), according to the guidelines of the EU registered Serbian Laboratory Animal Science Association (SLASA).

Seven weeks old male Wistar rats (250 ± 10 g), obtained from the local colony and separate litters, were maintained under standard conditions: group-housed (4 per cage) with free access to food (commercial pellet) and tap water, regular 12 h light/12 h dark cycle and constant temperature ($21 \pm 2^\circ\text{C}$) and humidity.

Treatment and surgical procedure

The experimental design is presented in Table 1. On the first day of the experiment, animals were randomly divided into two main groups: (I) control animals; (II) rats intragastrically intubated (reusable stainless steel feeding needle, 16-G4", 3 mm ball diameter, Cadence Inc., Staunton, Virginia, USA), three times per week for 4 weeks with butylated hydroxyanisole (BHA) (2% BHA in the powdered feed) (Sigma-Aldrich Co., Saint Louis, Missouri, USA). Although it has been reported that rat pellets may be enriched with BHA and the animals consume this food readily [3], animals refused to consume pellets prepared in this way, rapidly losing their body mass, thus forcing us to feed the animals by intubation a mixture of pellets with BHA. Additionally, two days prior to sacrifice animals were subdivided into experimental groups according to implemented BrdU doses: 50 mg, 100 mg, 200 mg or 350 mg ($n = 4$ per experimental group). Animal weight at this point was 372 ± 21 g. Flat-faced cylindrical matrices containing 50 mg of BrdU (Sigma-Aldrich Co., St. Louis, USA) with a diameter of 5.6 mm were prepared by a direct compression technique using an eccentric tablet press (Korsch EK-0, Korsch, Berlin, Germany). BrdU matrices were subcutaneously implanted in the dorsal neck region under 5% chloral hydrate (400 mg/kg, Sigma-Aldrich Co., St. Louis, USA) anesthesia.

Table 1. Number of BrdU positive cells in the forestomach epithelium treated with different doses of BrdU.

Groups	Doses of BrdU (mg)	Number of cells/mm ²
Control	200	18.24 ± 8.4
BHA	50	16.95 ± 6.1^a
BHA	100	51.07 ± 25.0^b
BHA	200	$67.63 \pm 21.5^*$
BHA	350	$75.27 \pm 21.0^*$

The results are represented as mean \pm SD

* $p < 0.001$ BHA treated vs. control animals; ^a $p < 0.01$ BHA treated animals which received 50 mg vs. animals which received 100, 200 and 350 mg BrdU; ^b $p < 0.01$ BHA treated animals which received 100 mg BrdU vs. animals which received 200 and 350 mg BrdU.

During post-operative recovery, physical health condition of all rats was closely monitored on a daily basis.

Tissue preparation

The animals were sacrificed by decapitation, the stomachs removed, the forestomach separated, cut along the minor curvature, washed in 0.9% aqueous sodium chloride, spread on paraffin substrate with pins and covered with 10% neutral buffered formalin for one week. After fixation, the tissues were dehydrated in a series of ethanol solutions (70%, 96% and 100%) and xylene, and then embedded into paraffin blocks. Paraffin tissue blocks of the forestomach were cut transversely into serial sections 5 μm in thickness.

Two consecutive sections, with ten levels, ranging from 250 μm between each level, were taken for: a) histochemical staining with hematoxylin and eosin for histological analysis and b) immunohistochemical staining for the detection of BrdU incorporation into DNA, i.e. analysis of cell proliferation. The analysis was done on light microscope Olympus AX70 with 10 \times and 20 \times objective magnification.

Immunohistochemical procedure

After deparaffinization in xylene, treatment with descending concentrations of alcohols, and rehydration in distilled water the forestomach sections were stained immunohistochemically according to the manufacturer's instructions (BrdU *in situ* detection kit, BD Pharmingen, New Jersey, USA). The procedure is based on the use of a directly biotinylated monoclonal antibody against BrdU, streptavidin-HRP solution as the detection system and 3,3'-diaminobenzidine (DAB, BD Pharmingen, New Jersey, USA) as a chromogen for visualization of BrdU incorporation. Forestomach tissue sections not treated with the primary antibody were used as the control. Counterstaining with Mayer's hematoxylin (Merck, Billerica, MA, USA) was used for the analysis on a light microscope.

Morphometry

Quantification of labeled (BrdU⁺) cells in the forestomach epithelium was performed using a computer-supported imaging system connected to a light microscope (Olympus AX70) with an objective magnification of 10 \times . The epithelial area was calculated according to the following formula:

$$P = p \times d^2 / 10^6,$$

Where, P is the surface area, p is the number of grid points in the epithelium, and d is the size of the square network at a magnification of 200 (10 \times objective). The number of BrdU⁺ cells/ mm^2 of epithelium (N) was calculated according to the formula:

$$N = n/P:$$

Here, n is the number of BrdU⁺ cells on the analyzed surface, and P is the analyzed surface area of epithelium. The number of proliferating (BrdU⁺) cells was expressed per mm^2 of epithelium.

Statistical analysis

The results are expressed as the mean \pm SD. Values were compared using the nonparametric Mann–Whitney U test in the program SPSS 10 for Windows. Differences at $p < 0.05$ were accepted as significant.

RESULTS

The results showed the presence of proliferating BrdU labeled cells (BrdU⁺) in the basal layer of the rat forestomach epithelium of the control and BHA treated animals (Figure 1). BHA treatment (2% in the powdered feed, three times per week) leads to

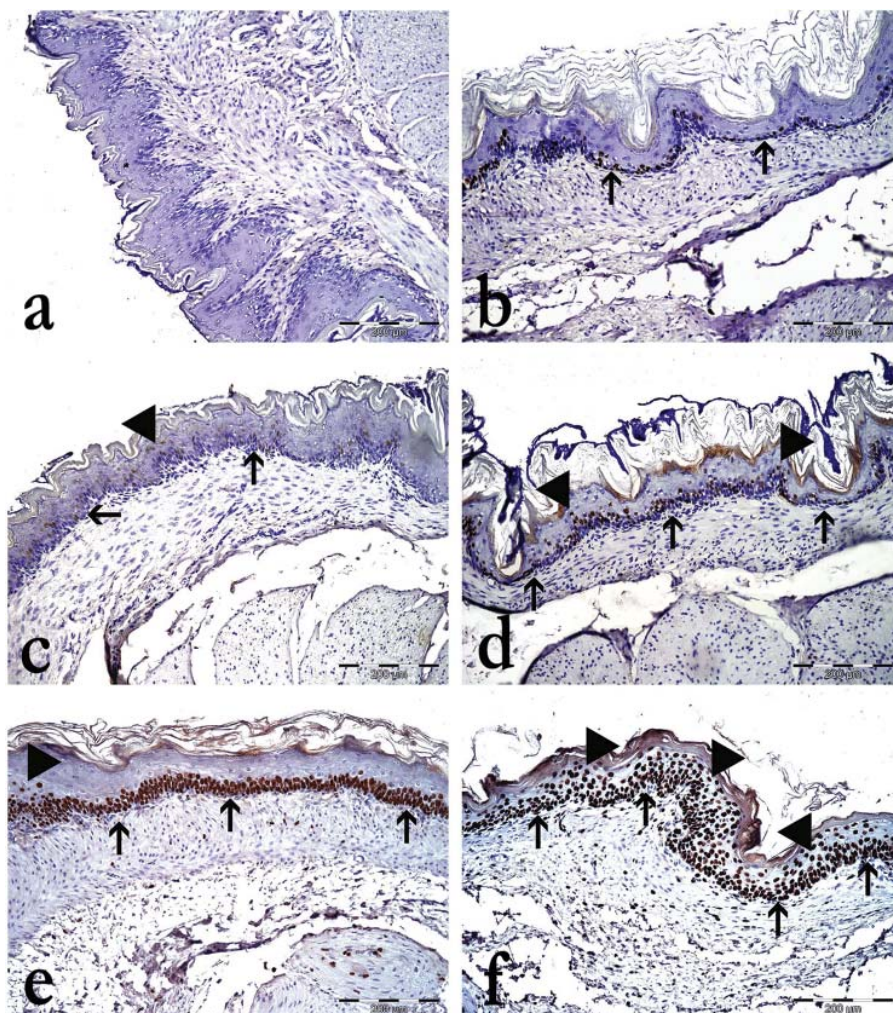


Figure 1. Immunohistochemical expression of BrdU positive cells in the rat forestomach epithelium. **(A)** Negative control. **(B)** Control rats treated with 200 mg BrdU. **(C-F)** Rats treated with the same doses of BHA and different doses of BrdU for 2 days: **(C)** 50 mg of BrdU, **(D)** 100 mg of BrdU, **(E)** 200 mg of BrdU and **(F)** 350 mg of BrdU. Scale bar 200 μ m (right angle down). Legend: arrow - basal layer; arrow head – zone of the granulosa.

the proliferation of cells of the basal layer of the epithelium, while the number of BrdU⁺ cells depends on the dosage of BrdU (Figure 1C-F). BHA-treated animals which received 50 mg of BrdU had proliferating cells mainly present in the basal layer of the epithelia and rarely in the zone of the granulosa (Figure 1C). In the treated animals, which had BrdU administered at a concentration of 100 and 200 mg a significant increase of BrdU⁺ cells could be seen in the basal layer of the epithelium arranged in several layers (Figure 1D-E). BHA treated animals which received 350 mg of BrdU had, in addition to the presence of labeled cells in the basal layer of the epithelium, an increased presence of the BrdU⁺ cells in the zone of the granulosa (Figure 1F).

Table 1. shows the number of BrdU⁺ cells in the epithelium of the control and BHA treated animals in which different doses of BrdU were administered. It was shown that in animals treated with BHA after the application of 50 mg of BrdU, the number of BrdU⁺ cells was not significantly changed in comparison to the control animals. However, application of higher doses of BrdU (100, 200 and 350 mg) significantly increased the number of BrdU⁺ cells in relation to the control and the group receiving 50 mg of BrdU. It was also shown that application of high doses of BrdU (200 and 350 mg) significantly increased the number of labeled cells in comparison to a dose of 100 mg of BrdU. There were no significant changes in the number of BrdU⁺ cells in the animals that received 200 mg compared to animals that received 350 mg of BrdU.

DISCUSSION

The use of BrdU for studying cell proliferation in various tissues is not of a recent date. The gut, kidney and liver were among the first analyzed tissues [13]. There are many described manners for BrdU *in vivo* labeling, treatment duration, methods of application and dose regimens. Therefore, the main objective of this study was to determine the optimum dose and application method of BrdU required for successful monitoring of cell proliferation in the rat forestomach for pharmacological and toxicological investigations. The rat forestomach was the target tissue considering its function as a storage organ. It serves as a holding compartment, so the tissue suffers prolonged exposure to xenobiotics [2]. In our study, butylated hydroxyanisole (BHA) was used as a proven cell proliferation agent [3]. Agents like BHA cause chronic inflammation or local irritation of the forestomach mucosa eventually leading to cell proliferation and hyperplasia [14]. *In vivo* labeling of DNA using BrdU should provide simple administration with minimal stress to animals and adequate dose regimens to ensure confident marking of cells.

Cantoreggi et al. (1993) applied BrdU *via* subcutaneous (s.c.) implantation of osmotic minipumps for proliferation studies [3]. Staszkiwicz et al. (2009) injected BrdU i.p. in mice twice daily for three consecutive days; Zhang et al. (2015) also used i.p. injection of BrdU twice a day at 2 h intervals over four consecutive days, whereas Ševc et al. (2015) administered BrdU dissolved in drinking water over a prolonged period of time [15-17]. While Staszkiwicz et al. (2009) and Zhang et al. (2015) concluded that

i.p. injections of BrdU is appropriate as a biomarker of their study, Ševc *et al.* (2015) concluded that oral administration of BrdU does not provide accurate information about the number of proliferating cells in target tissues [15-17]. Erben *et al.* (2008) reported subcutaneous implementation of BrdU pellets for 3 and 7 weeks exposure, however long term exposure resulted in BrdU toxicity accompanied with decreased labeling efficiency over time, thus the precise monitoring of cell proliferation can not be achieved in this manner [18]. Weghorst *et al.* (1991) showed that s.c. BrdU administration for 4 days provides more accurate data of the true proliferative scenario in cell kinetic studies in contrast to the pulse-labeled approach [19]. A further increase in treatment duration decreased the number of labeled cells. Comparative use of osmotic minipumps and slow-release BrdU pellets showed that nuclear labeling was very similar with both techniques. We found that implementation of BrdU matrices subcutaneously, two days prior to sacrifice provided a slow and continuous release similar to the effect of osmotic minipumps. In contrast to osmotic minipumps administration of BrdU matrices is simple, the wound is minimal and sutured with one surgical stitch.

The concentration of BrdU has an effect on cell proliferation across a wide range of doses depending on the way of application (40–480 mg/kg for i.p., and up to 2.5 g/kg for subcutaneous application) as reported in the literature [19,20]. Our study demonstrated that s.c. application of 200 mg of BrdU per animal is optimal for adequate labeling of proliferating cells in adult rats.

CONCLUSIONS

Our results showed that doses of 50 and 100 mg of BrdU administered subcutaneously are not sufficient for adequate labeling of proliferating cells of the rat forestomach. Subcutaneous administration of 200 mg of BrdU per animal (i.e. on average 540 mg of BrdU/kgBW) is optimal for labeling proliferating cells, whereas a further increase in BrdU dose does not have an impact on the number of labeled cells. Due to the fast turnover of rat forestomach epithelium the duration of BrdU exposure of two days gave sufficient results.

Evidently, trying to standardize the dose and application method of BrdU should simplify cell proliferation studies. At least in the case of adult rats, it may be accepted that the optimum BrdU dose is 200 mg per animal for s.c. application. These results do not provide only certain and unambiguous immunohistochemical results, but also reduces stress to animals as multiple injections are obliterated and elaborate minipumps are not required.

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Authors' contributions

JG and DM participated in the design of the study and performed the statistical analysis. NR conceived of the study, and participated in its design and coordination and helped to draft the manuscript. MM, FJ, DD, SM, ČB and VŠA carried out the immunoassays. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PRAĆENJE ĆELIJSKE PROLIFERACIJE – OPTIMIZACIJA METODE ZA *IN VIVO* OBELEŽAVANJE DNK ĆELIJA ŽELUCA PACOVA

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Praćenje ćelijske proliferacije je važno za istraživanja u oblasti toksikologije, patofiziologije i farmakologije. Kao pokazatelj stepena ćelijske proliferacije koristi se 5-bromo-2'-deoksiuridin koji se ugrađuje u nosivnu materiju DNK ćelija u S fazi ćelijskog ciklusa. Cilj ove studije je optimizacija potkožne implantacije 5-bromo-2'-deoksiuridina za kontinuirano i dugotrajno obeležavanje ćelija u deobi na modelu neželjanog dela želuca pacova. Ćelijska proliferacija indukovana je 3-tert-Butil-4-hidroksianizolom. 5-bromo-2'-deoksiuridin u dozama od 50 mg, 100 mg, 200 mg i 350 mg je implantiran dva dana pre žrtvovanja u formi pljosnatih cilindričnih matrica u cilju utvrđivanja optimalne doze za obeležavanje ćelija u deobi. Imunohistohemijska

analiza 5-bromo-2'-deoksiuridin in situ detekcionim kitom je korišćena za kvantifikaciju 5-bromo-2'-deoksiuridin obeleženih ćelija. Rezultati su pokazali da je za odrasle pacove optimalna doza 5-bromo-2'-deoksiuridina za potkožnu implantaciju 200 mg po životinji. Opisani način 5-bromo-2'-deoksiuridin in vivo obeležavanja omogućava jednostavan, efikasan i pouzdan način obeležavanja ćelija sa minimalnim stresom za životinje.