

TRANSDERMAL DELIVERY OF ENDOGENOUS GROWTH INHIBITING PROTEINS USING POLYVINYL ALCOHOL BASED FILMS

Khvedelidze N.Sh., Markarashvili E.G., Esatia I.GH., Otiashvili D.B., Dzidziguri D.V.

Ivane Javakhishvili Tbilisi State University, Tbilisi, Georgia
Nodar.khvedelidze106@ens.tsu.edu.ge

Abstract: To evaluate the feasibility of non-invasive, specifically transdermal, delivery of a thermostable growth – inhibiting protein complex (TPC) isolated from adult rat liver, we investigated its effects on cell proliferation in the tissues of juvenile rats when immobilized in polyvinyl alcohol (PVA)-based films of different compositions. It was found that immobilization of TPC in films composed of polyvinyl alcohol and vinyltriethoxysilane (VTES) led to a significant reduction in the mitotic index of liver and kidney cells in experimental animals compared with controls (by 46% and 30%, respectively). In contrast, immobilization of TPC in films composed solely of polyvinyl alcohol did not produce an inhibitory effect in any tissue. In parallel, we examined whether the incorporation of starch as a third component could enhance film solubility and the release of proteins. The results showed that starch addition did not increase film solubility, nor did it alter the antiproliferative activity of the immobilized TPC.

Key words: transdermal delivery, polyvinyl alcohol, vinyltriethoxysilane, thermostable protein complex (TPC).

Introduction

It is well known that various routes of drug administration exist, including oral, intravenous, intramuscular, and subcutaneous delivery; however, each of these routes is associated with specific limitations. These include the low stability of the drug in the gastrointestinal tract and its susceptibility to first-pass metabolism. In addition, drugs may undergo degradation due to enzymatic activity or the acidic environment of the stomach [1]. These and other limitations are particularly pronounced when administering peptide- or protein-based drugs [2]. Therefore, the search for alternative routes for delivering protein compounds into the body is highly relevant. One such route is the transdermal delivery of bioactive substances.

Transdermal drug delivery (TDD) is a noninvasive or minimally invasive method that allows a certain amount of drug to pass through the epidermal layer of the skin by free diffusion or other means and continues to enter the systemic circulation at a controlled rate [3]. When substances are delivered via this route, the aforementioned limitations are largely avoided.

Among transdermal delivery systems, films are the most common and widely used form. The film-forming polymers can be applied alone or in combination with other polymers to produce films with the desired properties [4]. Accordingly, the aim of the present study was to evaluate the feasibility of delivering a thermostable protein complex (TPC), which inhibits cell growth, immobilized in polyvinyl alcohol-based films of varying composition, into the body.

Materials and Methods

Experiments were carried out on non-linear white juvenile rats (7 days old). Animals were housed under controlled conditions at a temperature of $25 \pm 2^{\circ}\text{C}$, relative humidity of $60 \pm 10\%$, with room air changes 12-18 times/hour, and a 14/10 h light/dark cycle.

The experimental material consisted of a thermostable protein complex (TPC) isolated from the liver tissue of adult rats using alcohol extraction method [5]. Protein concentration was determined using the Lowry method [6]. Components of the protein complex were identified via native polyacrylamide gel electrophoresis (current: 7 mA; voltage: 100 V), and gels were stained with silver nitrate [7].

Following characterization, the protein complex was immobilized in polyvinyl alcohol (PVA)-based films of varying composition. Films were prepared using a 7% PVA solution (Mw 85,000–124,000). Experimental films included vinyltriethoxysilane (VTES) and 3 mg of lyophilized TPC. For starch-containing films, 5% starch

was incorporated. In a second series, films were prepared similarly but without VTES. Films (50 μm thick) were cut into 15 equal squares (1.5 cm^2), each containing approximately 130 μg of immobilized TPC.

To evaluate the effect of TPC immobilized in the films on cell proliferation, liver and kidney tissues were collected from juvenile rats. Two hours prior to tissue collection, control and experimental animals received colchicine injections (1 mg/kg). Tissues were fixed in 4% formaldehyde prepared in phosphate buffer (pH 7.4). Following standard processing, 5 μm -thick sections were prepared and stained with hematoxylin-eosin for microscopic analysis. At least 5000 cells were counted per sample, and the mitotic index was calculated per 1000 cells and expressed in per mille (‰). Each experimental group included a minimum of 12 animals.

In parallel, proliferative activity was also evaluated by Ki-67 immunohistochemistry. After deparaffinization, sections underwent antigen retrieval in citrate buffer, blocking with goat serum, and incubation with Ki-67 antibody (Abcam, 1:100). Detection was performed with biotinylated secondary antibody, DAB, and Mayer's hematoxylin counterstain. Ki-67-positive cells were quantified per 1000 cells (‰).

Data were analyzed using IBM SPSS software. One-way ANOVA was applied to assess differences in mitotic activity across tissue types. Post hoc comparisons were performed using Tukey's HSD test. Results are expressed as mean $\pm$ SEM. Differences were considered statistically significant at $P < 0.05$, with confidence levels ranging from 95% to 99%.

Results

At the initial stage of the study, native electrophoretic analysis of the growth-inhibitory thermostable protein complex (TPC) isolated from adult white rat liver was performed. The protein complex was found to contain high-molecular-weight (25–100 kDa) and low-molecular-weight (10–20 kDa) subfractions (Figure 1A). Notably, the low-molecular-weight component (14–17 kDa) has been shown to suppress cellular mitotic activity through transcriptional inhibition [8].

Initially, we assessed whether PVA/VTES films without immobilized protein affected mitotic activity in the liver and kidney tissues of juvenile rats. The analysis indicated no statistically significant differences compared to intact controls, with mitotic indices of $5.56 \pm 0.15\text{‰}$ versus $5.22 \pm 0.15\text{‰}$ in the liver ($p = 0.631$) and $5.52 \pm 0.37\text{‰}$ versus $5.55 \pm 0.31\text{‰}$ in the kidney ($p = 0.999$) (Fig. 1B). In contrast, in the experimental group treated with PVA/VTES films containing the thermostable protein complex (TPC), mitotic activity significantly decreased in both liver ($3.02 \pm 0.25\text{‰}$, $p < 0.00005$) and kidney ($3.92 \pm 0.20\text{‰}$, $p < 0.005$) tissues (Fig. 1B). Compared to intact controls, the mitotic index was reduced by 46% in the liver and by 30% in the kidney.

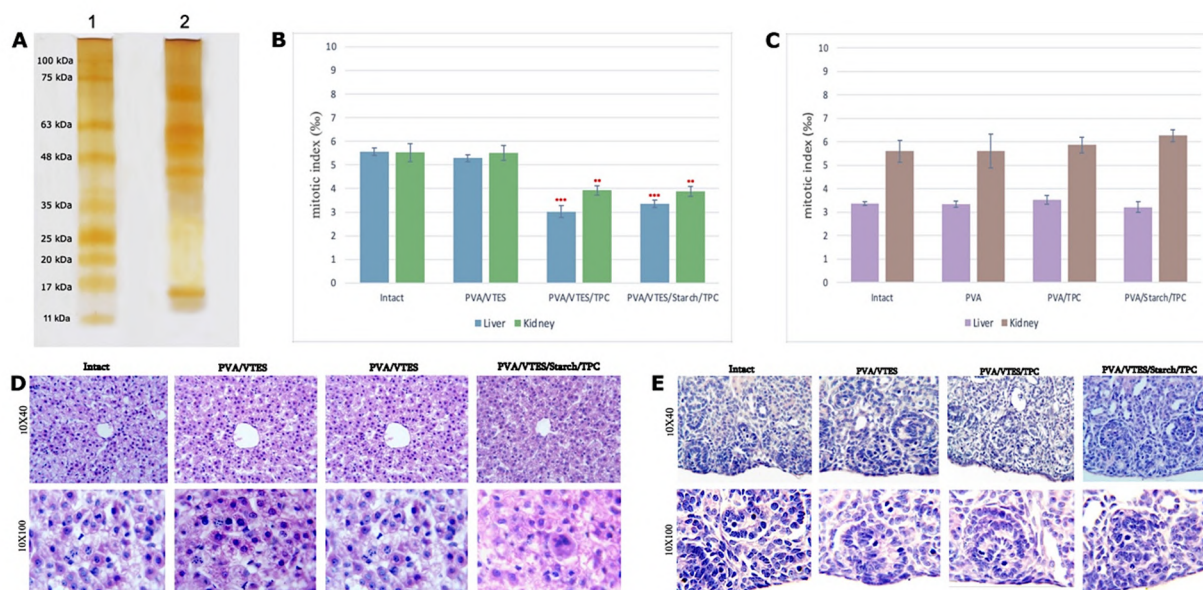


Fig. 1. (A) Native electrophoresis of proteins in a polyacrylamide gradient gel (10–25%): 1 – protein markers; 2 – thermostable protein complex (TPC) from adult white rat liver. (B) Effect of TPC immobilized in PVA/VTES films on mitotic activity in liver and kidney. (C) Effect of TPC immobilized in PVA films on mitotic activity in liver and kidney. (D) Liver histoarchitecture in control and experimental groups. (E) Kidney histoarchitecture in control and experimental groups.

To further evaluate the effect of TPC immobilized in the film on proliferative activity, we performed immunohistochemical analysis using antibodies against Ki-67, a marker of proliferating cells (Fig. 2). Analysis showed that in intact animals, the number of Ki-67-positive cells was $4.06 \pm 0.16\%$ in the liver (Fig. 2A) and $6.56 \pm 0.47\%$ in the kidney (Fig. 2B). After treatment with TPC-immobilized films, Ki-67-positive cells increased to $8.02 \pm 0.21\%$ in the liver and $11.54 \pm 0.25\%$ in the kidney.

To exclude any potential adverse effects of the polymer and TPC on the morpho-functional activity of cells, we evaluated the impact of the polyvinyl alcohol polymer on the histoarchitecture of liver and kidney tissues of white rats.

Histological examination showed that in both control and experimental animals, hepatocytes in the centrilobular region exhibited cytoplasmic vacuolization, reflecting normal physiological development and glycogen accumulation. The liver parenchyma contained numerous hematopoietic foci, which are characteristic of normal liver up to postnatal day 10 (PND10) [9]. No structural alterations were observed in the portal triads or hepatic sinusoids. Furthermore, no excessive infiltration of circulating cells was detected, indicating the absence of inflammation or other pathological processes (Fig. 1D).

In kidney sections, the cortical regions adjacent to the subcapsular zone exhibited an active nephrogenic area containing proliferating cells, including immature glomeruli and other developing nephron structures. Centrally located glomeruli appeared more mature, reflecting the normal gradient of nephron development. In both control and experimental groups, no evidence of inflammation, necrosis, or fibrosis was observed in the kidney tissue (Fig. 1E).

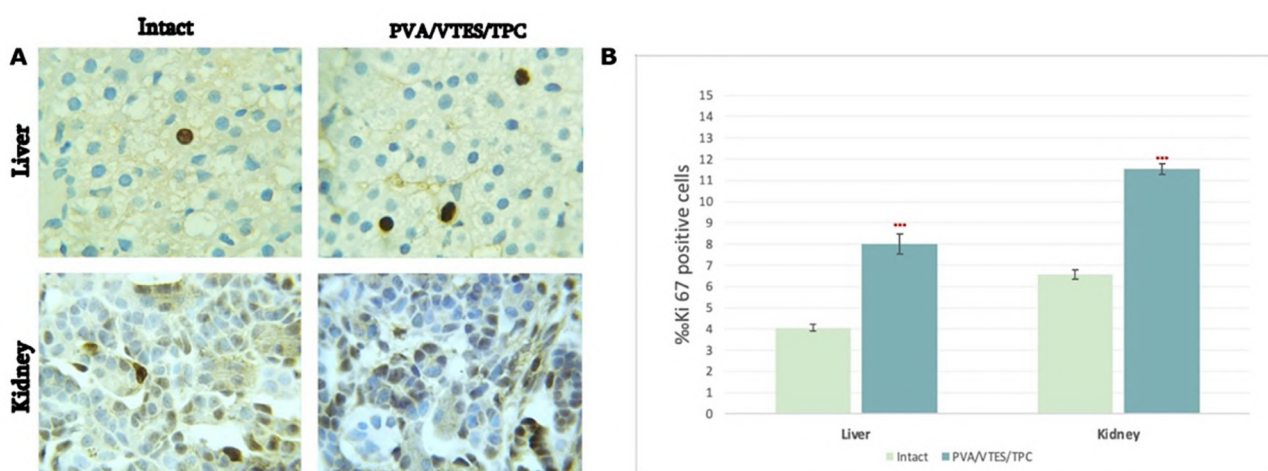


Fig. 2. Immunohistochemical staining using Ki-67 antibodies. (A) Histological sections of liver and kidney from control and experimental animals (10×100). (B) Effect of TPC immobilized in PVA/VTES films on the number of Ki-67-positive cells in liver and kidney.

Additionally, to evaluate the influence of the components used in film preparation on film solubility, a second series of experiments was conducted using TPC immobilized in PVA films without VTES.

In intact animals, the mitotic index was $3.37 \pm 0.08\%$ in the liver and $5.6 \pm 0.46\%$ in the kidney. After treatment with TPC immobilized in PVA-only films, mitotic activity in the experimental group was not significantly altered: liver $3.53 \pm 0.17\%$ ($p > 0.05$) and kidney $5.87 \pm 0.33\%$ ($p > 0.05$) (Figs. 1C, 2B).

At the next stage of the study, we aimed to improve the properties of PVA-based films of different compositions. To achieve this, starch was used, as it is known from the literature to increase PVA film solubility, water retention, and environmental biodegradability [10], which could enhance the release of immobilized proteins from the film and, consequently, their biological effect.

The study showed that in PVA-only films, the addition of starch did not confer any antiproliferative effect of the immobilized TPC on liver or kidney tissues. In contrast, in PVA/VTES films, the addition of starch did not alter the antiproliferative effect of the immobilized TPC in these tissues.

Discussion

We have previously demonstrated that TPC inhibits cell proliferation through transcriptional suppression [8]. In the present study, we sought to determine whether this endogenous protein complex retains its biological activity *in vivo* when administered noninvasively via the transdermal route. For this purpose, PVA-based films were used.

To exclude any potential negative (toxic) effects of the polymer films on the morpho-functional activity of cells, we evaluated their impact on liver histoarchitecture in white rats. The results demonstrated that film application did not induce any histoarchitectural changes in the liver or kidney tissues of experimental animals, indicating that the observed reduction in mitotic activity was due to the specific biological action of the protein complex rather than polymer toxicity.

Compared to intact controls, treatment with TPC immobilized in PVA/VTES films resulted in a significant decrease in mitotic activity in both liver and kidney, suggesting that TPC is absorbed through the skin and reaches these organs, retaining its ability to suppress mitotic activity in juvenile rat tissues. In contrast, TPC immobilized in PVA-only films showed no effect on proliferative activity in liver or kidney, indicating that the presence of a crosslinker, in this case VTES, is essential for protein release from the film.

Furthermore, the addition of starch to PVA-only films did not confer antiproliferative activity of immobilized TPC, confirming that VTES is necessary for protein release. In PVA/VTES films, starch addition did not increase the antiproliferative effect of immobilized TPC, which may be due to the limited absorption rate through the skin despite enhanced release from the film.

Interestingly, in tissues of experimental animals, while the mitotic index decreased under TPC treatment, the number of Ki-67-positive cells increased, likely reflecting compensatory accelerated entry of cells into the cell cycle in the juvenile organism.

Conclusion

Based on the results of the present study, we can conclude that the feasibility of using a polyvinyl alcohol/vinyltriethoxysilane (PVA/VTES) film for non-invasive delivery of biologically active substances is directly dependent on the presence of vinyltriethoxysilane. Moreover, the use of starch as an additional component in a film based on polyvinyl alcohol cannot ensure an increase in the penetration rate of the thermostable protein complex through the film, and consequently, does not enhance its inhibitory effect.

References

1. Dahan A, Miller JM, Amidon GL., Prediction of solubility and permeability class membership: provisional BCS classification of the world's top oral drugs. // *AAPS J.* 11, 2009, 740–6.
2. Wen N.X., Venkatraman S. Protein delivery options: how well have we succeeded? *Ther Deliv.*, 6, 2015, pp. 537–539. doi: 10.4155/tde.15.11.
3. Long L., Zhang J., Yang Z., Guo Y., Hu X., Wang Y., Transdermal delivery of peptide and protein drugs: Strategies, advantages and disadvantages. *Journal of Drug Delivery Science and Technology*, 60, 2020, 102007.
4. Pünnel L.C., Lunter D.J., Film-Forming Systems for Dermal Drug Delivery. *Pharmaceutics*. 2021 Jun 23;13(7), 2021, p. 932. doi: 10.3390/pharmaceutics13070932. PMID: 34201668; PMCID: PMC8308977.
5. Balazs A, Blazsek I., Control of cell proliferation by endogenous inhibitors. *Akademia Kiado (Budapest)* 1979;302
6. Lowry D.H., Rosenbrough N.J., Farr A.L., Randell R.J., Protein measurement with the folin phenol reagent. // *J. Biol. Chem.*, vol. 193, 1951, pp. 265-275.
7. Davis B., *J. Ann. New York Acad. Sci* 121, 1964, p. 404.
8. Dzidziguri D, Modebadze I, Bakuradze E, Mosidze G, Berulava M. Determination of The Properties of Rat Brain Thermostable Protein Complex which Inhibit Cell Proliferation. *Cell J.* 2018 Jan;19(4), 2018, pp. 552-558. doi: 10.22074/cellj.2018.4835. Epub 2017 Nov 4. PMID: 29105389; PMCID: PMC5672093
9. Parker, G. A., Picut, C. A., Hofbauer, C. S. (Eds.). *Atlas of histology of the juvenile rat.* Academic Press, 2016.
10. Negim, EL-Sayed & Urkimbaeva, P.I. & Rakhmetullayeva, Raikhan & S.T., Primzharova & Yeligbayeva, Gulzhakhan & Kaldybekov, Daulet & Khatib, Jamal & Mun, Grigoriy & Williams, Craig. Improving Biodegradability of Polyvinyl alcohol/Starch Blend films for Packaging Applications. *International Journal of Basic and Applied Sciences*, 3, 2014 pp. 263-273. doi:10.14419/ijbas.v3i3.2842.