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Article: Gold nanoparticles carrying or not anti-VEGF antibody do not change glioblastoma multiforme tumor progression in mice

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Supplemental material

1. Material and methods

1.1 PVDF membrane protein staining

To measure the anti-VEGF Ig conjugation process, we stained proteins in a polyvinylidene fluoride membrane (PVDF) (GE Healthcare, Little Chalfant, Burckinghamshire, United Kingdom), after performing electrophoresis in polyacrylamide gel, either in presence of cit-AuNP or anti-VEGF Ig-covered AuNP once it was reported greater affinity of conjugated AuNP for proteins in a PVDF membrane [1-3]. In brief, frozen cerebral tumor was powered, homogenized in lysis buffer (10% RIPA buffer, Merk Millipore, MA, USA), added 1 mM phenylmethylsulfonyl fluoride (PMSF) (Amresco, Solon, OH, USA), 10 mM sodium orthovanadate (Sigma-Aldrich, St Louis, MO, USA), 100 mM sodium fluoride (LabSynth, Diadema, SP, Brazil), 10 mM sodium pyrophosphate (Sigma-Aldrich), and 0.2% protease inhibitor (P8340, Sigma-Aldrich), for 30 min at 4ºC, and centrifuged (15.000x g, 4ºC, 20 min) to obtain supernatant. Total proteins were quantified in supernatant (PierceTM BCA Protein Assay Kit, ThermoFisher

Scientific) and treated with Laemmli's buffer containing 350 mM dithiothreitol. Fifty micrograms of proteins were loaded on a 15% polyacrylamide gel (Sigma-Aldrich) and transferred to a polyvinylidene fluoride membrane (PVDF) (GE Healthcare, Little Chalfant, Burckinghamshire, United Kingdom) for 40 min, 80V and 4^{o}C. Nonspecific binding was blocked using 3% BSA in tween Tris buffered saline (TTBS) buffer (pH 7.6) (LabSynth) for 1 h, at room temperature. Membranes were incubated overnight with either anti-VEGF-A-covered Ig AuNP or cit-AuNP (Nitparticles Company, Zaragoza, Spain) at 4°C. Membranes were put in presence of a quimoluminescence solution (Pierce® ECL Western Blotting Substrate, Thermo Fisher Scientific) for better distinction between membranes, and images captured using a luminescence reader device (Carestream Molecular Imaging, Gel Logic 2200 PRO, Carestream Health, NY, EUA).

1.2 Immunoprecipitation

To determine whether the gold nanoparticle conjugation process affected or not the ability of the antibody to bind VEGF, we performed an immunoprecipitation assay followed by western blotting. To do that we used 500 micrograms of cerebral homogenate proteins, obtained as described in the main text – western blotting section –, which were diluted (1:10, v:v) in phosphate buffered saline – PBS – and incubated overnight in fridge either with anti-VEGF Ig-covered AuNP or cit-AuNP (300 µL each). Nanoparticles were precipitated by centrifugation (13,000 rpm, 10 min), pellet and supernantant (pos-IP) obtained. Pellet was washed in 0.1% tween 20-PBS (TPBS) and resuspended by sonication (Fisher Scientific, model 100, Waltham, MA, USA). This washing step was repeated three times. To separate the

VEGF-antibody-gold nanoparticles complex, pellet was resuspended in Laemmli's buffer containing 350 mM dithiothreitol (Sigma-Aldrich), and boiled for 5 minutes. Same procedure was performed for pos-IP sample. Vials containing samples were put in ice and VEGF was quantified by western blotting the same protocol described in the main text.

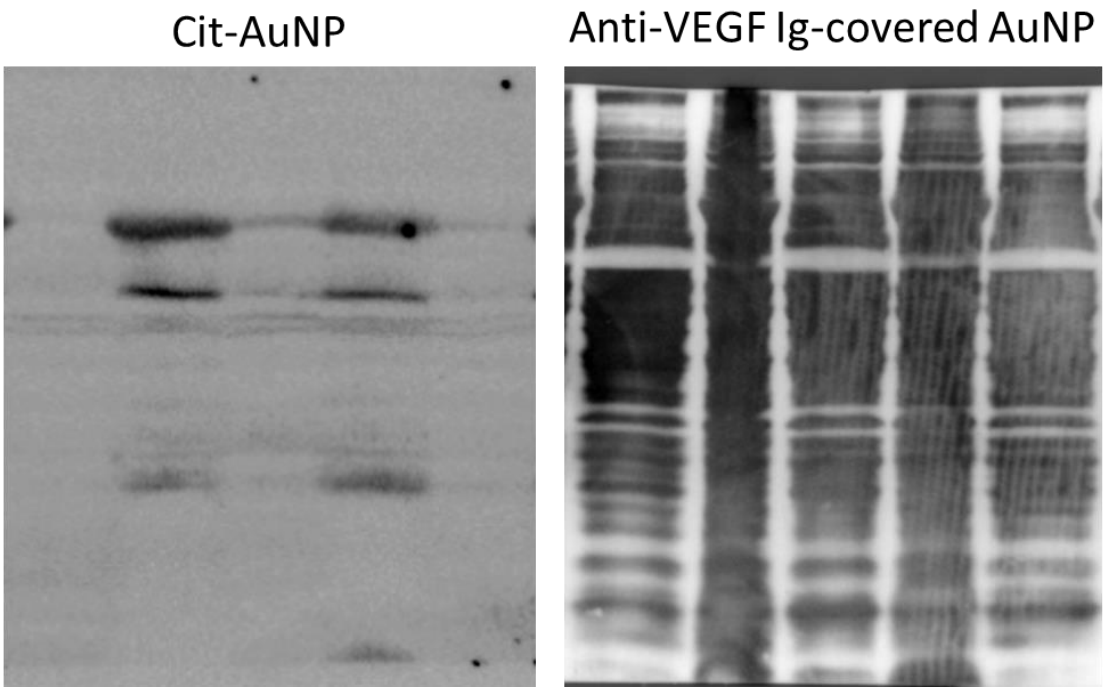
1.3 Quantifying clotted blood vessels in the tumor areas

To quantify the clotted blood vessels represented by the red color in the cerebral tumor sections from mice chronically treated anti-VEGF Ig-covered AuNP, anti-VEGF Ig or saline we used the Image J program (Image J 1.47v, NIH, USA). First, we informed the program a known distance in each photo, converted the image to grayscale and split images into the three original colors (red, blue and green). After that, we adjusted threshold for correcting the intensity of red, and quantified just the remaining red dots area that were representative of the clotting blood vessels in the tumor.

2. Results

2.1 Anti-VEGF Ig-AuNP conjugation process confirmation

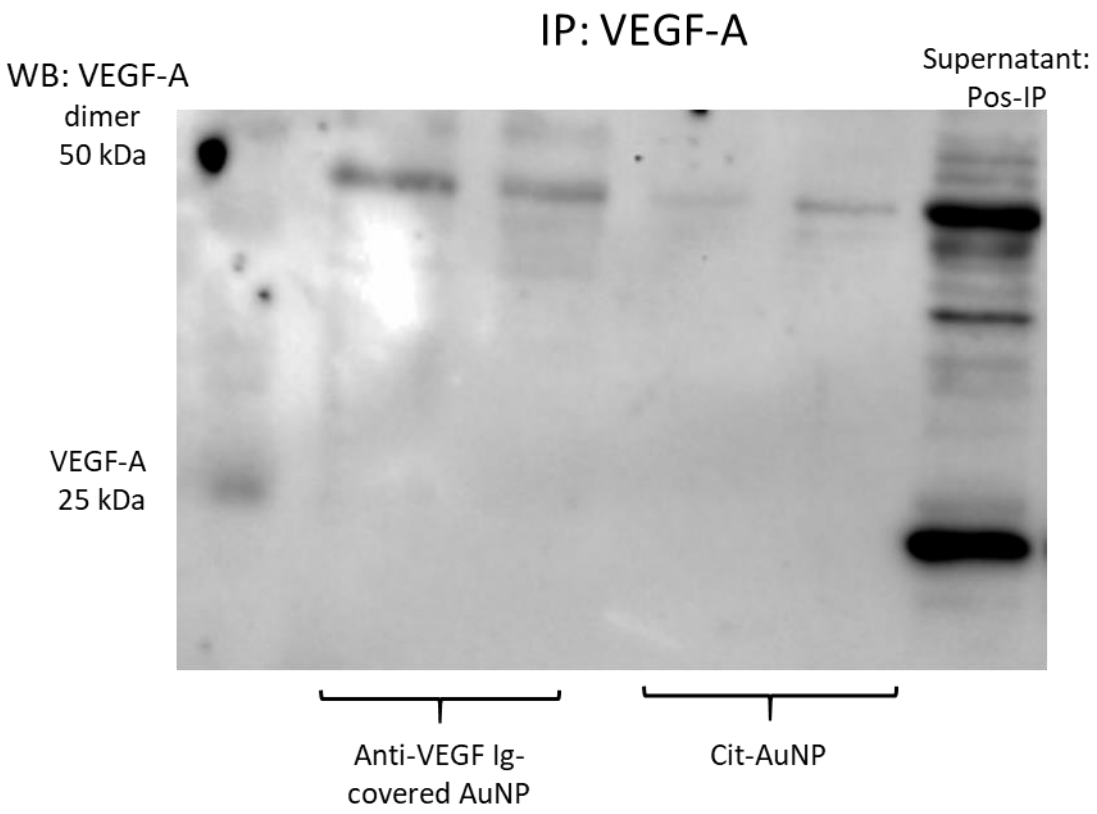
Incubation of the same cerebral samples with anti-VEGF Ig-covered AuNP resulted in a more pronounced labeling of proteins dispersed in a PDVF membrane compared to cit-AuNP incubation (Supplementary Figure 1).



Supplementary figure 1. Proteins labeling in PVDF membranes incubated either with anti-VEGF Ig-covered AuNP or cit-AuNP. Greater protein labeling was visualized in the PVDF membrane overnight incubated with anti-VEGF Ig-covered AuNP (right side panel) compared to cit-AuNP incubation (left side panel). Same protein samples and quantity were used for incubation with anti-VEGF Ig-covered AuNP and cit-AuNP.

2.2 Anti-VEGF Ig-AuNP functional activity confirmation

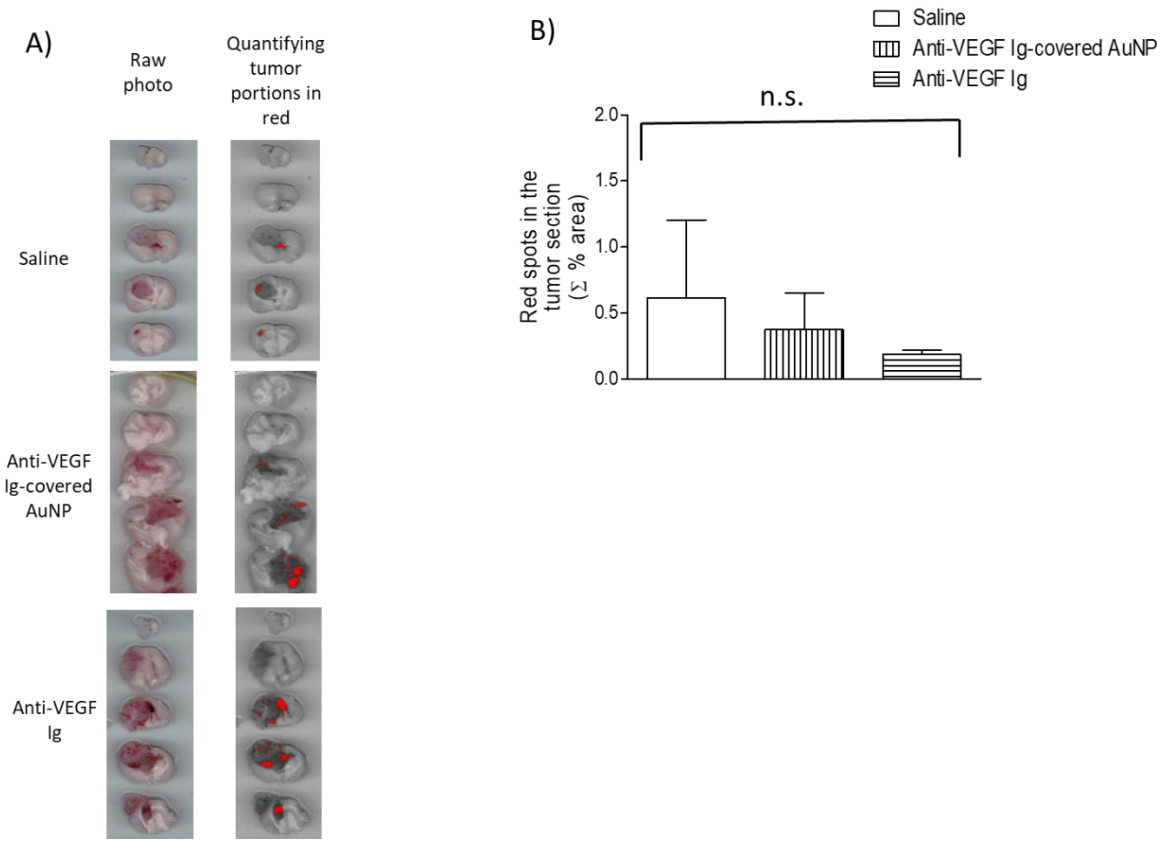
By doing immunoprecipitation of VEGF with the anti-VEGF Ig-AuNP followed by VEGF detection by western blotting, we confirmed anti-VEGF Ig was functional after conjugation once the anti-VEGF Ig-AuNP recognized the VEGF dimer present in the cerebral proteins homogenate of mice, as can be observed in the supplementary figure 2. An anti-VEGF Ig preference for the dimer VEGF over the monomer structure was previously reported [4].



Supplementary figure 2. Anti-VEGF activity after gold nanoparticle conjugation. Anti-VEGF Ig-covered AuNP was used to immunoprecipitate (IP) VEGF-A from a cerebral protein homogenate sample obtained from mice. Western blotting (WB) for VEGF-A was performed to visualize the immunoprecipitated protein. VEGF-A in their common monomer and dimer forms are visualized in the supernatant sample, obtained after sample centrifugation following immunoprecipitation. Anti-VEGF Ig-covered AuNP preference for the VEGF-A dimer form over monomeric form was noticed. Small amount of VEGF-A was precipitated by cit-AuNP.

2.3 Clotted blood vessels in the tumor sections

Both chronic anti-VEGF Ig-covered AuNP and anti-VEGF Ig did not significantly change clotted blood vessels in the tumor compared to saline treated mice (Supplementary figure 2).



Supplementary figure 3. Effect of anti-VEGF antibody (Ig)-covered gold nanoparticles, anti-VEGF Ig or saline on the clotted blood vessels in the GL261 cells-induced glioblastoma multiforme (GBM) in mice. A) Representative raw (left) or highlighted portions in red (right) of 2 mm-cerebral sections obtained from GBM-carrying mice intravenously and chronically treated with anti-VEGF Ig-covered-AuNP, anti-VEGF Ig or saline (control groups) from day 8 up to day 25 after GBM induction, injected once every three days. B) Red spots (clotted blood vessels)

were quantified in tumor sections from each group and no difference was observed among those groups ($P > 0.05$). Three animals per group were assayed and ANOVA followed by Tuker test were used for comparison. Not significant, n.s.

References

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