International Journal for Parasitology 52 (2022) 377–383

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Proliferative cells in racemose neurocysticercosis have an active MAPK signalling pathway and respond to metformin treatment

Miguel A. Orrego [a,*], Manuela R. Verastegui [b], Carlos M. Vasquez [c], Hector H. Garcia [a,d], Theodore E. Nash [a], for the Cysticercosis Working Group in Peru

[a] Laboratory of Immunopathology in Neurocysticercosis, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Avenida Honorio Delgado 430, Urb. Ingenieria, SMP. Lima 31, Peru
[b] Infectious Diseases Research Laboratory, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Avenida Honorio Delgado 430, Urb. Ingenieria, SMP. Lima 31, Peru
[c] Department of Neurosurgery, Instituto Nacional de Ciencias Neurológicas, Jirón Ancash 1270, Barrios Altos, Lima 01, Peru
[d] Cysticercosis Unit, Instituto Nacional de Ciencias Neurológicas, Jirón Ancash 1270, Barrios Altos, Lima 01, Peru

ARTICLE INFO

Article history:
Received 6 September 2021
Received in revised form 29 December 2021
Accepted 3 January 2022
Available online 17 February 2022

Keywords:
Cisticercosis
Neurocysticercosis
Subarachnoid cyst
Taenia solium
MAPK

ABSTRACT

Racemose neurocysticercosis is an aggressive infection caused by the aberrant expansion of the cyst form of *Taenia solium* within the subarachnoid spaces of the human brain and spinal cord, resulting in the displacement of the surrounding host tissue and chronic inflammation. We previously demonstrated that the continued growth of the racemose bladder wall is associated with the presence of mitotically active cells but the nature and control of these proliferative cells are not well understood. Here, we demonstrated by immunofluorescence that the racemose cyst has an active mitogen-activated protein kinases (MAPK) signalling pathway that is inhibited after treatment with metformin, which reduces racemose cell proliferation in vitro, and reduces parasite growth in the murine model of *Taenia crassiceps* cysticercosis. Our findings indicate the importance of insulin receptor-mediated activation of the MAPK signalling pathway in the proliferation and growth of the bladder wall of the racemose cyst and its susceptibility to metformin action. The antiproliferative action of metformin may provide a new therapeutic approach against racemose neurocysticercosis.

1. Introduction

Neurocysticercosis (NCC) is the infection of the CNS by larval cysts of the tapeworm *Taenia solium* (Garcia et al., 2003). Infection in the intermediate host (human, pig) occurs after ingestion of eggs containing the hexacanth embryo or oncosphere (Pawlowski, 2002). The eggs hatch in the small intestine and the released oncospheres penetrate the intestinal mucosa, enter the bloodstream, and lodge within the organs. Although cysts can be found in any blood-supplied organ, mature cysts develop mostly in the subcutaneous tissue, muscle, and brain (Pawlowski, 2002; Garcia et al., 2003). Mature vesicular cysts consist of a fluid-filled vesicle containing an invaginated scolex.

Clinical manifestations are due to parasite burden, cyst location and degree of inflammation (Nash and Garcia, 2011). Treatment consists of the anti-helminthc agents albendazole or praziquantel, as well as corticosteroids or other anti-inflammatory agents (Nash and Garcia, 2011). Parasites located in the subarachnoid spaces, particularly in the basal cisterns or in the Sylvian fissures, are characterised by continuous growth to form a multivesicular membranous structure known as a racemose cyst (Marcin Sierra et al., 2017). The ability of the racemose cyst to proliferate has profound implications for disease evolution and treatment (Nash et al., 2006; Nash et al., 2020).

Several studies previosly demonstrated the importance of host factors such as transforming growth factor (TGF) β, epidermal growth factor (EGF), fibroblast growth factor (FGF), or insulin in the development and proliferation of cestodes (Spiliotis et al., 2003; Escobedo et al., 2009; Adalid-Peralta et al., 2017; Förster et al., 2019). However, the causes or mechanisms associated with the development and abnormal growth of the racemose cyst are poorly understood. We previously identified proliferative cells in the bladder wall of the racemose cyst (Orrego et al., 2021) which were stimulated to proliferate in vitro by insulin (Orrego et al., 2021).

The insulin signalling pathway has critical impacts on many biological processes, including glucose uptake, glycogen synthesis, growth, and cell proliferation (Boucher et al., 2014). The main components of the insulin signalling pathway have been identified in

* Corresponding author.
E-mail address: miguel.orrego.s@upch.pe (M.A. Orrego).

https://doi.org/10.1016/j.ijpara.2022.01.001
0020-7519/

several platyhelminths (Wang et al., 2014). Insulin signalling is initiated by binding of insulin-like hormones to surface receptor tyrosine kinases of the insulin/insulin-like growth factor (IGF) family (Boucher et al., 2014). Upon ligand binding to surface-associated α2β2 receptor tetramers, auto-phosphorylation of several tyrosine residues within the β-subunit is induced (Sun et al., 1993; Benyoucef et al., 2007).

Rabbits immunised with the ligand-binding domain of *T. solium* insulin receptor conferred protection against infection with cystic *Taenia pisiformis* (Wang et al., 2020), confirming the importance of the insulin pathway in the life cycle of the parasite and its potential use as a therapeutic target.

The two major downstream insulin signalling pathways in vertebrates and invertebrates are the extracellular signal-regulated protein kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) cascade and the phosphoinositide-3-kinase (PI3K)/protein kinase B (PKB; also known as Akt) pathway (Boucher et al., 2014).

Metformin (N, N-dimethylbiguanide), an antihyperglycemic agent widely used in type 2 diabetes mellitus treatment, is effective against cancer and highly proliferative parasites such as *Echinococcus multilocularis* and *Echinococcus granulosus* (Loos et al., 2017, 2020). The antiproliferative activity of metformin is partially attributed to the activation of 5′ AMP-activated protein kinase (AMPK) with suppression of mammalian target of rapamycin (mTOR) as well as ERK1/2 (Li et al., 2020).

Here, we demonstrate that the proliferation of the racemose cyst is associated with the activation of the MAPK signalling pathway mediated by phosphorylation of the insulin receptor (IGFR). We also show, in vitro and in vivo, that metformin treatment inhibits proliferation of the bladder wall of the *T. solium* racemose cyst and *Taenia crassiceps* cysts by targeting proliferative cells.

2. Materials and methods

2.1. Experimental design

Bladder wall samples from vesicular and racemose *T. solium* cysts were used to compare the expression levels of insulin receptor 1 and 2 by quantitative PCR (qPCR), as well as to evaluate phosphorylation levels in the MAPK signalling pathway by in situ immunofluorescence. Additionally, the in vitro action of metformin was evaluated in proliferative cells isolated and cultured from the racemose bladder wall. Finally, we employed the murine *T. crassiceps* cysticercosis model to evaluate, in vivo, the effectiveness of metformin to inhibit parasite proliferation.

2.2. Parasite sample collection

Portions of racemose cysts discarded after surgery on patients with subarachnoid NCC were collected, anonymized, and transported to the laboratory in PBS pH 7.4. The viability of all bladder wall samples was assessed by double staining with methylene blue and mitotracker as previously described (Orrego et al., 2021). Only viable sections were selected for the study. The use of anonymized racemose NCC samples was approved by the Ethics Committee of Instituto Nacional de Ciencias Neurológicas (INCN) in Lima, Peru (242-2018-DG-INCN).

Samples of vesicular cysts were removed from the skeletal muscle of infected pigs and transported to the laboratory in PBS pH 7.4 (Gibco-Invitrogen, Gaithersburg, MD) supplemented with antibiotics (100 U/ml of penicillin and 100 g/ml of streptomycin from Gibco-Invitrogen, Gaithersburg, MD, USA). For this, naturally infected pigs were bought from endemic cities of Peru, transported to the veterinary facilities of Universidad Nacional Mayor de San Marcos (UNMSM) in Lima, Peru, and humanely euthanized (Gonzales et al., 2012). Pig infection was grossly established by positive tongue examination, and confirmed by the demosnstration of specific antibodies on enzyme linked immunoelectrotransfer blot (EITB) assay.

For the murine *T. crassiceps* cysticercosis model, 6 week old female BALB/c mice were housed in facilities at the bioterium of Universidad Peruana Cayetano Heredia (UPCH), Lima, Peru and inoculated intraperitoneally with 10 cysts suspended in RPMI medium. After 3 months, the animals were euthanized with chloroform and abdominal cysts were collected (Robinson et al., 2002). All protocols for the use of animals were approved by the Institutional Ethics Committees for the Use of Animals of the Veterinary School of UNMSM (Protocol number 006) and UPCH (Protocol numbers 62392 and 101250).

2.3. Sample processing

Sections of both types of cyst bladder walls were either fixed in neutral buffered formalin and paraffin-embedded for histology, used for cell isolation, or preserved in RNAlater (Qiagen) at −70 °C for total RNA isolation.

2.4. Generation of cDNA and qPCR for insuline receptor 1 and 2

qPCR was performed on six vesicular and six racemose cyst samples. The bladder wall and scolex from each vesicular cyst were separated manually with a scapel for total RNA isolation. They were homogenised in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for standard RNA isolation and concentrations were determined using a UV spectrophotometer (Nanodrop Products, Wilmington, DE, USA). cDNA was generated from 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit with MultiScribe RT polymerase and random primers (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 µl per reaction and incubation for 10 min at 25 °C followed by 60 min at 37 °C, 5 min at 95 °C on a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA). qPCR was performed in 10 µl reaction volumes using SsoAdvanced Universal SYBR Green Supermix (BioRad Laboratories, Hercules, CA, USA) with primers designed for insulin receptor 1 (MT242602.1) (Forward: 5′-AGAGGTTG CAGGCGTTAAA-3′) (Reverse: 5′-CACGGAACCAGAGAAGATACAC-3′) , insulin receptor 2 (MT242603.1) (Forward: 5′-GAACGAGCCTAACAGTCCTAAC-3′) (Reverse: 5′-GCTGCGTCATCCTC CAAATA-3′) (Wang et al., 2020), and *gapdh* genes (TsM_000056400) (Forward: 5′-TCCAAGAGATGAATGCCAATGC-3′) (Reverse: 5′-CAGAAGGAGCCGAGATGATGA-3′) (Logan-Klumpler et al., 2012). qPCRs, run in triplicate, used the following cycling parameters: pre-incubation of 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, on a Lightcycler 96 System (Roche, Basel, Switzerland). The bladder walls of vesicular cysts were used as a calibration sample and we expressed the results as relative to the expression of the gapdh gene using the $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001).

2.5. Bioinformatic analysis

The amino acid sequences of insulin receptor and ERK 1/2 from *T. solium*, *E. multilocularis*, *Mus musculus*, and *Homo sapiens* were selected from the GenBank and GeneDB databases, and analysed by BLAST. Conserved phosphorylation sites were identified by multiple alignments with CLUSTALW. Additionally, the presence of functional domains was identified with Pfam. All bioinformatic tools are available at www.genome.jp.

2.6. Immunofluorescence (IF) for phospho-IGF receptor and phospho-Erk1/2

The formalin-fixed paraffin-embedded (FFPE) samples of seven racemose and 12 vesicular cysts were cut into 4 μm sections placed on poly-L-lysine coated slides and thereafter processed. The slides were submerged in 10 mM citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) for 30 min at 95 °C, incubated for 30 min with a blocking solution (PBS pH 7.2, 0.05% Tween 20, 0.1% Triton X-100, 2% goat serum, 2% BSA) in a humid chamber at room temperature, then incubated overnight at 4 °C with rabbit anti-phospho-IGF receptor (Tyr1135/1136)/(Tyr1150/1151) antibody (Cell Signalling Technology, Danvers, MA, USA; 1:250 dilution) or rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody (Cell Signalling Technology; 1:250 dilution). The sections were washed three times for 2 min with washing solution (PBS pH 7.2, 0.05% Tween 20) and then incubated for 30 min at room temperature with the fluorescein-labelling goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; 1:500 dilution). The sections were then washed with PBS and mounted with VectaShield mounting medium with DAPI (Vector, Laboratories, Burlingame, CA, USA). Images were captured by confocal microscopy (Zeiss, LSM880, Oberkochen, Germany). Five images per condition of three independent replicates were randomly selected, and the number of phospho-IGF receptor/phospho-ERK1/2 positive cells and total nuclei were counted. Normal goat serum was used as a negative control.

2.7. Proliferative cell isolation and culture conditions

The sections of racemose larvae selected for cellular isolation were processed as previously described (Orrego et al., 2021). Briefly, the samples were treated with a solution of 0.025% trypsin and 0.01% EDTA, incubated for 15 min at 37 °C, and the tube was shaken very gently for 3 min. The supernatant was collected and 35 ml of PBS were added to the samples and shaken vigorously for 3 min. The supernatant was collected again and the process was repeated. The three collected fractions were pooled and then centrifuged for 10 min at 400 *g*, the supernatant was discarded and the pellet was resuspended with RPMI 1640 medium (supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml of penicillin, 100 g/ml of streptomycin and 0. 25g/ml of amphotericin B, 0.01 mM non-essential amino acids, 0.2 mM L-glutamine, 1.6 μM β-mercaptoethanol, 25 mM HEPES; all from Gibco-Invitrogen, Gaithersburg, MD, USA). The cells were cultivated in 12-well plates at 37 °C and 5% CO_2.

2.8. In vitro effects of metformin on membrane sections and in cultured proliferative cells

Membrane sections and previously harvested, growing proliferative cells were used to evaluate the effects of metformin. Three racemose cyst viable sections (each one 5 mm), randomly selected, were cultured with supplemented RPMI 1640 medium for 2 weeks in the presence of metformin (4 mM), then treated and untreated sections were processed for immunofluorescence studies to evaluate the direct effects of metformin on the MAPK signalling pathway in the racemose cyst bladder wall.

Also, the cells of three primary cultures were resuspended and viability was evaluated with trypan blue dye using a hemocytometer. We seeded 200,000 cells isolated from a T. solium racemose cyst and T. crassiceps cysts in 24-well plates. Three concentrations of metformin (0.4, 4, and 40 mM), and untreated cells as controls, were evaluated in triplicate and cell counts were performed every 24 h.

2.9. In vivo efficacy of metformin treatment on murine T. crassiceps cysticercosis

Six-week-old female mice infected with 10–20 cysts of *T. crassiceps* (ORF strain) were randomly assigned into two groups: six mice received metformin (50 mg/kg/day) as a suspension in water administered orally by gavage for 3 months and four mice received water alone (controls). At the end of the treatment, the animals were euthanized with chloroform, the peritoneal cavity opened, the cysts carefully collected, and their total weight determined for each animal. The efficacy of treatments was calculated using the following formula: 100 × [(mean cyst weight of control group) − (mean cyst weight of treated group)]/ (mean cyst weight of control group) according to Loos et al. (2017).

2.10. Statistical analysis

Non-parametric statistics (Mann-Whitney U test for two groups) were calculated using Prism software (Graphpad, San Diego, CA, USA) for comparisons of the gene expression between bladder walls of both types of cysts (racemose and vesicular), and for in vivo metformin effects in the murine *T. crassiceps* cisticercosis model. Differences with P values <0.05 were considered statistically significant. The ratios of positive cells by IF to nuclei fluorescence were calculated and the growth curves in cell proliferation assays were formatted as a dot plot with the mean ± S.D. All quantitative comparisons of in vitro metformin effects were analysed by one-way ANOVA and P values <0.05 were considered statistically significant.

3. Results

3.1. Taenia solium has highly conserved MAPK pathway proteins

To determine the percentage identity of the insulin receptor and ERK 1/2, we performed a BLAST analysis. The insulin receptor 1 (QKL20108.1) and 2 (QKL20109.1) of *T. solium* showed a high percentage of identity (83%) with *E multilocularis* (AJ458426.1; HG326255.1) and 32% to 49% with *H. sapiens* (AH002851.2). In addition, ERK 1/2 (TsM_000447200) showed 95% identity with *E. multilocularis* (EmuJ_000891000.1; EmuJ_000803700.1) and 69% with *H. sapiens* (NP_002736.3).

Multiple sequence alignment revealed that the primary amino acid sequences of the insulin receptor (Supplementary Fig. S1) and ERK1/2 (Supplementary Fig. S2) of *T. solium* are highly conserved compared with *E. multilocularis*, *H. sapiens* and *M. musculus* for threonine and tyrosine residues. Furthermore, these sequences show a domain structure typical of receptor tyrosine kinase and protein kinase, respectively (Supplementary Figs. S3, S4).

3.2. Expression levels of the insulin receptor are increased in the racemose cyst of Taenia solium

Expression of the two insulin receptor isoforms was determinated by qPCR using specific primers. The expression of both isoforms was significantly increased in the racemose cyst compared with the vesicular cyst (median: 4.5 for racemose versus 1.0 for vesicular, P value <0.01, Mann-Whitney U test) (Fig. 1).

3.3. The racemose cyst of Taenia solium showed an active MAPK pathway

Using racemose and vesicular cyst tissue samples, we evaluated by IF the phosphorylation levels of the MAPK signalling pathway. The number of phospho-IGFR positive cells was significantly higher

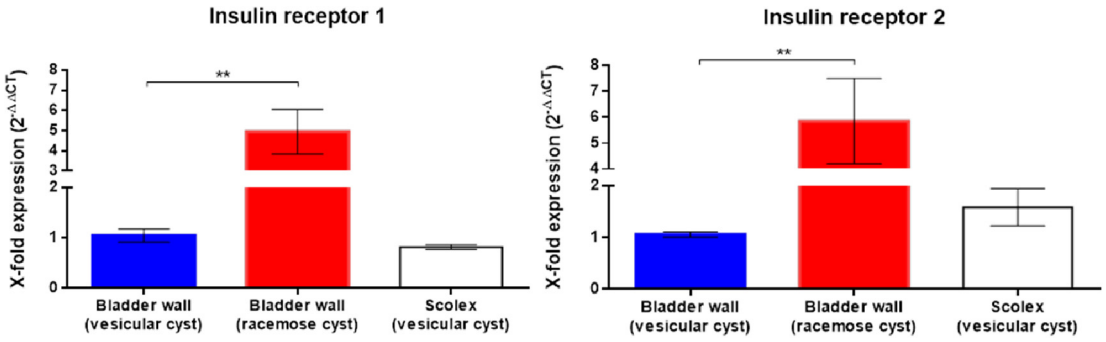

Fig. 1. Quantitative PCR for insulin receptor 1 and 2 in *Taenia solium* cysts. Quantitative PCR of cDNA from 500 ng of total RNA from the racemose form compared with the bladder wall and scolex of the vesicular form. Results are normalised to the housekeeping *gapdh* gene and expressed as the fold increase in expression. Statistically significant differences in leves of gene expression are indicated by asterisks (Mann-Whitney U test). $^{**}P < 0.01$.

in the bladder wall of the racemose cyst compared with the vesicular cyst (mean: 626.7 cells for racemose versus 0 cells for vesicular, *P* value <0.0001, one-way ANOVA test) (Fig. 2B); these cells were completely absent in the vesicular form (Fig. 2E). Next, we evaluated the phosphorylation levels of downstream protein ERK1/2. As in the previous assay, we observed positive cells only in the bladder wall of the racemose cyst (mean: 104.3 cells for racemose versus 0 cells for vesicular, *P* value <0.001, one-way ANOVA test) (Fig. 3B, E).

3.4. Metformin reduces cell proliferation

We evaluated the direct effects of metformin on the racemose cyst bladder wall. Sections were cultivated for 2 weeks in the presence of metformin to evaluate the phosphorilation levels of IGFR and ERK1/2 post-treatment by IF. While metformin (4 mM) had no effect on the phosphorylation levels of IGFR (Fig. 2H) (mean: 650 cells for treated racemose versus 626 cells for untreated racemose, one-way ANOVA test), it significantly reduced the phosphorylation levels of ERK 1/2 in treated sections compared with untreated (mean: 41.3 cells for treated racemose versus 104.3 cells for untreated racemose, *P* value < 0.05, one-way ANOVA test) (Fig. 3H).

To evaluate the effects of metformin on proliferative cells, we used bladder wall sections of the racemose cyst and *T. crassiceps* cyst to establish proliferative cell cultures following the protocol previously described (Orrego et al., 2021). Proliferative cells were cultured in the presence of three different concentrations of metformin. We observed that after 24 h, metformin at 4 mM and 40 mM significantly reduced proliferation in *T. solium* (mean: 270,000 cells for control versus 188,333 cells for 4 mM and 155,000 cells for 40 mM, *P* value <0.001; *P* value <0.0001, one-

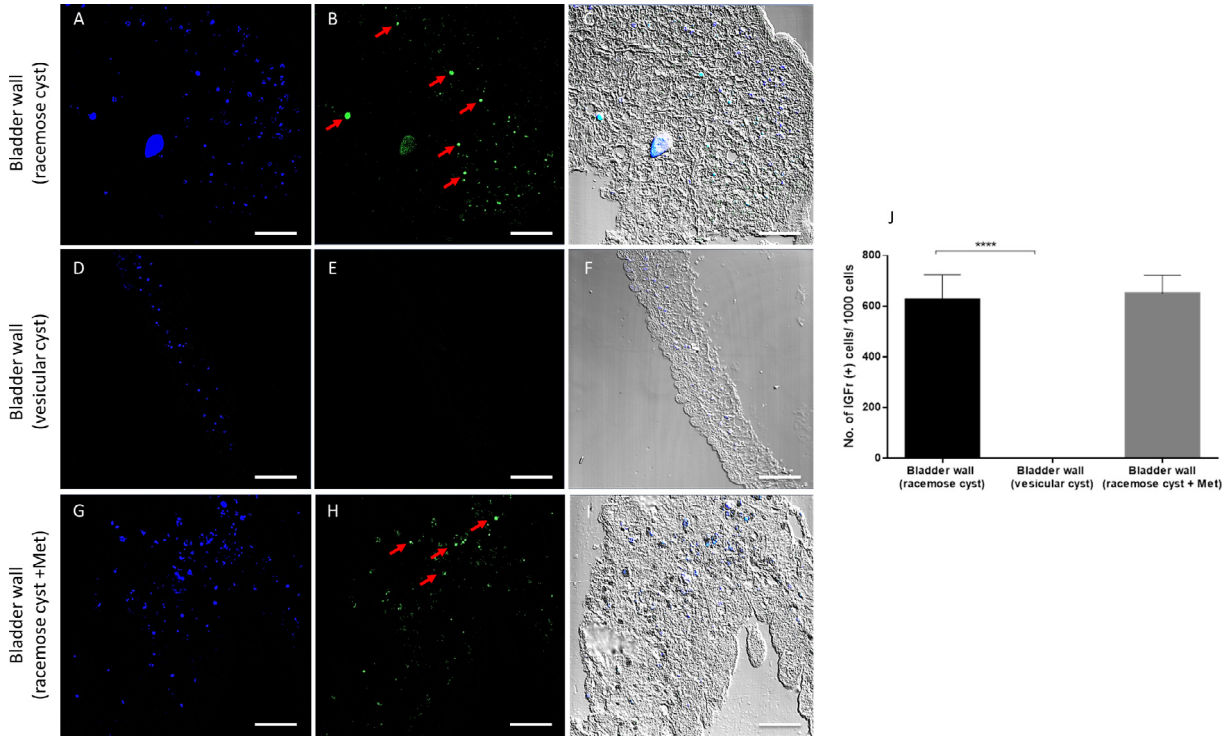

Fig. 2. In situ identification of activated Insulin-like Growth factor receptor (IGF) in *Taenia solium* cysts. Immunofluorescence performed on a racemose cyst, vesicular cysts, and racemose cyst treatment with metformine (Met). Nuclei were stained with DAPI (A, D, G). Cells positive for the phospho IGF receptor (B, E, H) were observed only in racemose larvae (red arrows). Merge (C, F, I). Representative images. The graph (J) depicts the ratio of phospho IGF to nuclei fluorescence intensity in racemose cyts, untreated and treated with Met (4 mM) relative to vesicular cysts. Values are expressed as means ± S.D. One-way ANOVA test ($^{****}P < 0.0001$).

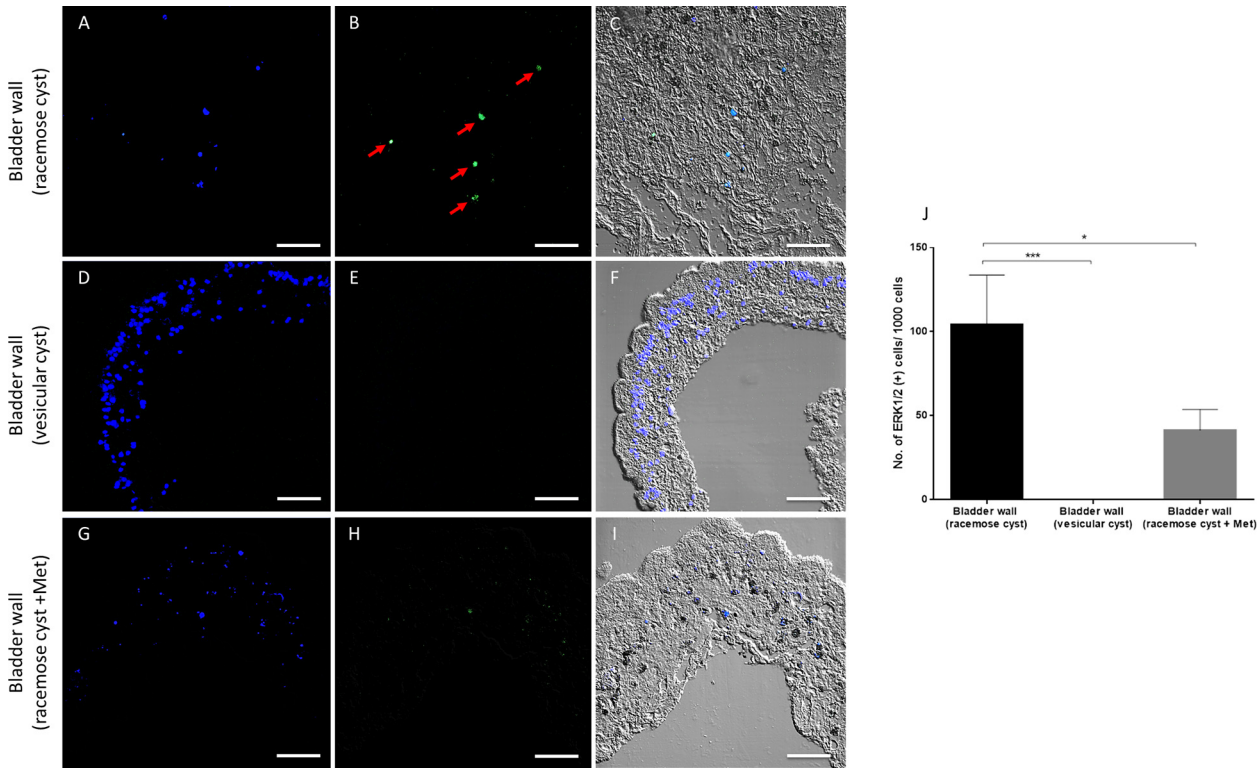

Fig. 3. In situ identification of activated extracellular signal-regulated kinase 1/2 (ERK 1/2) in *Taenia solium* cysts. Immunofluorescence performed on a racemose cyst, vesicular cysts, and racemose cyst treatment with metformine (Met). Nuclei were stained with DAPI (A, D, G). Cells positive for phospho ERK1/2 (B, E, H) were observed only in racemose larvae (red arrows). Merge (C, F, I). Representative images. The graph (J) depicts the ratio of phospho ERK1/2 to nuclei fluorescence intensity in racemose cyts, untreated and treated with Met (4 mM), relative to vesicular cysts. Values are expressed as means ± S.D. One-way ANOVA test (*$P < 0.05$; ***$P < 0.001$).

way ANOVA test) and *T. crassiceps* cell cultures (mean: 250,000 cells for control versus 188,000 cells for 4 mM and 150,000 cells for 40 mM, *P* value <0.01; *P* value <0.001, one-way ANOVA test) (Fig. 4A, B).

Finally, we evaluated the effecacy of metformin treatment in vivo, using the murine *T. crassiceps* cysticercosis model. Cysts of the ORF strain proliferate asexually by budding in the peritoneal cavity of mice. Infected mice received metformin (5 mg/kg/day) or

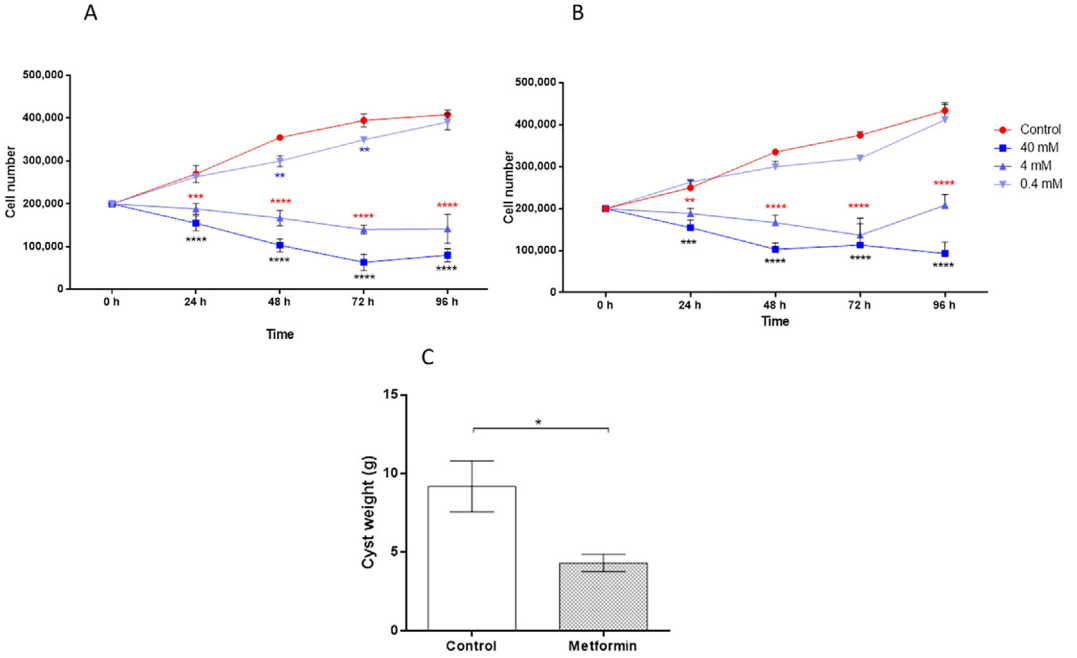

Fig. 4. In vitro and in vivo efficacy of metformin in *Taenia* spp. Growth curves of cells isolated from *Taenia solium* racemose larvae (A) and a *Taenia crassiceps* cyst (B). Metformin (Met) at 40 mM and 4 mM produced a significant decrease in cells after 24 h (red and black asterisks). Asterisks indicate statistically significant differences between groups (treated and control). One-way ANOVA test (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). The graph (C) shows the weight of *T. crassicpes* cysts recovered from untreated (control) and treated (Met 50 mg/Kg/day) mice. Mann-Whitney U test (*$P < 0.05$).

just water, orally for 3 months. During the treatment period, no alterations in food consumption were observed. Metformin treatment reduced the number of cysts and total weight by 53% (median: 8.594 g for control versus 4.331 g for treated, *P* value <0.05, Mann-Whitney U test) (Fig. 4C).

4. Discussion

Racemose NCC is considered the most aggressive form of NCC (Fleury et al., 2011), characterised by continuous growth, associated with the displacement of surrounding brain tissue and exacerbated local inflammation (Nash et al., 2020). The abnormal dimensions reached by the racemose cyst and its abnormal morphology suggest alterations in pathways associated with cell proliferation. The MAPK signalling pathway in organisms from vertebrates to invertebrates including cestodes plays a key role in the translation of extracellular signals to generate diverse cellular responses including cellular proliferation, differentiation, or development (Zhang and Liu, 2002; Cheng et al., 2017). Although conserved pathways have been identified in *T. solium* (Wang et al., 2014), their involvement in the proliferation of the racemose cyst is unknown. Our observations reveal for the first known time the presence of cells with active IGFR as well as the mediator ERK1/2. These results suggest the importance of IGFR-mediated activation of the MAPK/ERK signalling pathway in bladder wall proliferation of the racemose cyst of *T. solium*.

The insulin signalling pathway is highly conserved and plays a critical role in cell growth and metabolic regulation (Hemer et al., 2014; Guo et al., 2020). Previously, we identified proliferative cells in the bladder wall of the racemose cyst, and these cells were sensitive to insulin action, shortening their doubling time (Orrego et al., 2021). We showed that two isoforms of the insulin receptor are overexpressed in racemose cysts, which likely reflects their increased need for glucose and energy in proliferating cells compared with resting vesicular cysts. The biological role of vesicular cysts is to persist in the host and develop into a tapeworm after ingestion. Further studies are needed to identify the totality of metabolic alterations that occur in the racemose cyst.

Racemose larvae have properties similar to tumours, which in itself suggests abnormal growth (Trelles et al., 1952). Similar to tumours of higher animals, the racemose cyst shows alterations in gene expression and activation of the MAPK pathway. Considering that tumorigenesis occurs by the synergistic interaction of complex signalling pathways such as MAPK, PI3K/Akt/mTOR, Ras, and Myc (Steelman et al., 2008; Guo et al., 2020), we focused on evaluating only the MAPK pathway. Further studies are required to determine whether other pathways are also involved in development of the racemose cyst.

Proliferation of primary cell cultures of racemose and *T. crassiceps* cysts was inhibited by metformin, suggesting deleterious effects in vitro. These findings are consistent with results from pre-clinical cancer studies (Shi et al., 2017). In addition, sections of the bladder walls of racemose cysts exposed to metformin for 2 weeks resulted in a significant reduction in the number of cells positive for the active form of ERK 1/2.

Using the murine model of cysticercosis, we evaluated the action of metformin in vivo and observed a reduction in cyst weight and parasite burden. Considering that *T. crassiceps* cysts are characterised by high proliferation of the bladder wall, our results suggest a selective action of metformin on proliferative cells, reducing their proliferative rate and the formation of new vesicles.

Metformin has beneficial effects in several pathologies such as cancer (Zi et al., 2018), multiple sclerosis, seizures, and Alzheimer's disease (Rotermund et al., 2018). Studies using glioma cells show a reduction in proliferation and a significant increase in apoptosis after treatment with metformin (Xiong et al., 2019). Although increased apoptosis could lead to synaptic disruption and epileptogenesis, studies demonstrate a reduction in status epilepticus induced by metformin (Vazifehkhah et al., 2020; Bojja et al., 2021). In addition, metformin induces metabolic reprogramming in $CD8^+$ T cells, and enhances protection against *Mycobacterium tuberculosis* (Böhme et al., 2020). Our results demonstrate a significant reduction in cell proliferation in the racemose cyst in vitro and in vivo, however further studies are required to evaluate the possible metabolic changes induced in the parasite, as well as possible neuroprotective and anti-inflammatory effects of metformin in patients with subarachnoid NCC.

The concentrations of metformin used in our in vitro evaluations (40, 4, and 0.4 mM) were high compared with the concentrations obtained in plasma of patients after taking metformin (Tucker et al., 1981); however, they are lower than the concentrations used to evaluate the in vitro effects on cancer stem cells (Shi et al., 2017). Previous studies report that the concentrations of metformin needed to induce biological effects in vitro are higher than the plasma levels of metformin in vivo due to a possible accumulation of metformin in organs such as the intestine (Bailey et al., 2008; Proctor et al., 2008).

The vesicular cyst constitutes a non-proliferative latent stage, while the racemose cyst presents different cellular and metabolic characteristics, and drugs with other modes of action should be considered and tested for the treatment of racemose NCC. The use of metformin for the treatment of racemose NCC by targeting proliferative cells represents an attractive alternative that requires further evaluation, such as its use in combination with praziquantel or albendazole, which could improve the efficacy of current anthelmintic treatment.

Acknowledgements

The authors would like to thank Drs. William Martinez, William Lines, Luis Saavedra, and Jose Calderon for their expertise and support. This work was supported by the National Council for Science, Technology, and Technological Innovation (CONCYTEC) Peru – World Bank, through its executing unit National Fund for the Development of Science, Technology, and Technological Innovation (FONDECYT) Peru contract [E033-01-08-2018-FONDECYT/Banco Mundial-Programas de Doctorado en Áreas Estratégicas y Generales; Fogarty International Center-National Institutes of Health Training grant D43 TW001140. The Cysticercosis Working Group in Peru: Hector H. Garcia, MD, PhD; Robert H. Gilman, MD, DTMH; Armando E. Gonzalez, DVM, PhD; Manuela Verastegui, PhD; Mirko Zimic, PhD; Javier Bustos, MD, MPH; Seth E. O'Neal, MD, MPH, and Victor C. W. Tsang, PhD (Coordination Board); Silvia Rodriguez, MSc; Isidro Gonzalez, MD; Herbert Saavedra, MD; Sofia Sanchez, MD, MSc, Manuel Martinez, MD (Instituto Nacional de Ciencias Neurologicas, Lima, Peru); Saul Santivanez, MD, PhD; Holger Mayta, PhD; Yesenia Castillo, MSc; Monica Pajuelo, PhD; Gianfranco Arroyo, DVM, MSc; Nancy Chile, MSc; Luz Toribio; Miguel Angel Orrego, MSc (Universidad Peruana Cayetano Heredia, Lima, Peru); Maria T. Lopez, DVM, PhD; Luis Gomez, DVM; Cesar M. Gavidia, DVM, PhD, Ana Vargas-Calla, DVM, Eloy Gonzales, DVM (School of Veterinary Medicine, Universidad Nacional Mayor de San Marcos, Lima, Peru); Luz M. Moyano, MD; Ricardo Gamboa, MSc; Claudio Muro; Percy Vichez, MSc (Cysticercosis Elimination Program, Tumbes, Perú); Sukwan Handali, MD; John Noh (Centers for Disease Control, Atlanta, GA); Theodore E. Nash, MD (NIAID, NIH, Bethesda, MD); Jon Friedland (Imperial College, London, United Kingdom).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2022.01.001.

References

Adalid-Peralta, L., Rosas, G., Arce-Sillas, A., Bobes, R.J., Cárdenas, G., Hernández, M., Trejo, C., Meneses, G., Hernández, B., Estrada, K., Fleury, A., Laclette, J.P., Larralde, C., Sciutto, E., Fragoso, G., 2017. Effect of transforming growth factor-beta upon *Taenia solium* and *Taenia crassiceps* Cysticerci. Sci. Rep. 7 (1), 12345.

Bailey, C.J., Wilcock, C., Scarpello, J.H.B., 2008. Metformin and the intestine. Diabetologia 51 (8), 1552–1553.

Benyoucef, S., Surinya, K.H., Hadaschik, D., Siddle, K., 2007. Characterization of insulin/IGF hybrid receptors: contributions of the insulin receptor L2 and Fn1 domains and the alternatively spliced exon 11 sequence to ligand binding and receptor activation. Biochem. J. 403 (3), 603–613.

Böhme, J., Martinez, N., Li, S., Lee, A., Marzuki, M., Tizazu, A.M., Ackart, D., Frenkel, J. H., Todd, A., Lachmandas, E., Lum, J., Shihui, F., Ng, T.P., Lee, B., Larbi, A., Netea, M.G., Basaraba, R., van Crevel, R., Newell, E., Kornfeld, H., Singhal, A., 2020. Metformin enhances anti-mycobacterial responses by educating CD8+ T-cell immunometabolic circuits. Nat. Commun. 11 (1), 5225.

Bojja, S.L., Medhi, B., Anand, S., Bhatia, A., Joshi, R., Minz, R.W., 2021. Metformin ameliorates the status epilepticus-induced hippocampal pathology through posible mTOR modulation. Inflammapharmacology 29 (1), 137–151.

Boucher, J., Kleinridders, A., Kahn, C.R., 2007. Insulin receptor signaling in normal and insulin-resistant states. Cold Spring Harb Perspect. Biol. 6(1), a009191.

Cheng, Z., Liu, F., Li, X., Dai, M., Wu, J., Guo, X., Tian, H., Heng, Z., Lu, Y., Chai, X., Wang, Y., Brehm, K., 2017. EGF-mediated EGFR/ERK signaling pathway promotes germinative cell proliferation in *Echinococcus multilocularis* that contributes to larval growth and development. PLoS Negl. Trop. Dis. 11 (2), e0005418.

Escobedo, G., Romano, M.C., Morales-Montor, J., 2009. Differential *in vitro* effects of insulin on *Taenia crassiceps* and *Taenia solium* cysticerci. J. Helminthol. 83 (4), 403–412.

Fleury, A., Carrillo-Mezo, R., Flisser, A., Sciutto, E., Corona, T., 2011. Subarachnoid basal neurocisticercosis: focus on the most severe form of the disease. Expert. Rev. Anti. Infect. Ther. 9 (1), 123–133.

Förster, S., Koziol, U., Schäfer, T., Duvoisin, R., Cailliau, K., Vanderstraete, M., Dissous, C., Brehm, K., Siles-Lucas, M., 2019. The role of fibroblast growth factor signalling in *Echinococcus multilocularis* development and host-parasite interaction. Plos Negl Trop Dis. 13 (3), e0006959.

Garcia, H.H., Gonzalez, A.E., Evans, C.AW., Gilman, R.H., 2003. *Taenia solium* cysticercosis. Lancet 362 (9383), 547–556.

Gonzales, A.E., Bustos, J.A., Jimenez, J.A., Rodriguez, M.L., Ramirez, M.G., Gilman, R. H., Garcia, H.H., Cysticercosis Working Group in Peru, 2012. Efficacy of diverse antiparasitic treatments for cisticercosis in the pig model. Am. J. Trop. Med. Hyg. 87 (2), 292–296.

Guo, Y.J., Pan, W.W., Liu, S.B., Shen, Z.F., Xu, Y., Hu, L.L., 2020. ERK/MAPK signalling pathway and tumorigenesis. Exp. Ther. Med. 19 (3), 1997–2007.

Hemer, S., Konrad, C., Spiliotis, M., Koziol, U., Schaack, D., Förster, S., Gelmedin, V., Stadelmann, B., Dandekar, T., Hemphill, A., Brehm, K., 2014. Host insulin stimulates *Echinococcus multilocularis* insulin signalling pathways and larval development. BMC Biol. 12, 5.

Li, L., Wang, T., Hu, M., Zhang, Y., Chen, H., Xu, L.u., 2020. Metformin overcomes acquired resistance to EGFR TKIs in EGFR-mutant lung cancer via AMPK/ERK/NF-κB signaling pathway. Front Oncol. 10, 1605.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta CT) method. Methods 25 (4), 402–408.

Logan-Klumpler, F.J., De Silva, N., Boehme, U., Rogers, M.B., Velarde, G., McQuillan, J. A., Carver, T., Aslett, M., Olsen, C., Subramanian, S., Phan, I., Farris, C., Mitra, S., Ramasamy, G., Wang, H., Tivey, A., Jackson, A., Houston, R., Parkhill, J., Holden, M., Harb, O.S., Brunk, B.P., Myler, P.J., Roos, D., Carrington, M., Smith, D.F., Hertz-Fowler, C., Berriman, M., 2012. GeneDB—an annotation database for pathogens. Nucleic Acids Res. 40 (D1), D98–D108.

Loos, J.A., Dávila, V.A., Brehm, K., Cumino, A.C., 2020. Metformin suppresses development of the *Echinococcus multilocularis* larval stage by targeting the TOR pathway. Antimicrob. Agents Chemother. 64 (9), e01808–e1819.

Loos, J.A., Dávila, V.A., Rodrígues, C.R., Petrigh, R., Zoppi, J.A., Crocenzi, F.A., Cumino, A.C., Casulli, A., 2017. Metformin exhibits preventive and therapeutic efficacy against experimental cystic echinococcosis. PLoS Negl. Trop. Dis. 11 (2), e0005370.

Marcin Sierra, M., Arroyo, M., Cadena Torres, M., Ramírez Cruz, N., García Hernández, F., Taboada, D., Galicia Martinez, Á., Govezensky, T., Sciutto, E., Toledo, A., Fleury, A., 2017. Extraparenchymal neurocysticercosis: demographic, clinicoradiological and inflammatory features. PLoS. Negl. Trop. Dis. 11, (6) e0005646.

Nash, T.E., Garcia, H.H., 2011. Diagnosis and treatment of neurocysticercosis. Nat. Rev. Neurol. 7 (10), 584–594.

Nash, T.E., O'Connell, E.M., Hammoud, D.A., Wetzler, L., Ware, J.M., Mahanty, S., 2020. Natural history of treated subarachnoid neurocysticercosis. Am. J. Trop. Med. Hyg. 102 (1), 78–89.

Nash, T.E., Singh, G., White, A.C., Rajshekhar, V., Loeb, J.A., Proano, J.V., Takayanagui, O.M., Gonzalez, A.E., Butman, J.A., DeGiorgio, C., Del Brutto, O.H., Delgado-Escueta, A., Evans, C.A.W., Gilman, R.H., Martinez, S.M., Medina, M.T., Pretell, E.J., Teale, J., Garcia, H.H., 2006. Treatment of neurocysticercosis: current status and future research needs. Neurology 67 (7), 1120–1127.

Orrego, M.A., Verastegui, M.R., Vasquez, C.M., Koziol, U., Laclette, J.P., Garcia, H.H., Nash, T.E., Diaz, A., 2021. Identification and culture of proliferative cells in abnormal *Taenia solium* larvae: Role in the development of racemose neurocysticercosis. Plos Negl. Trop. Dis. 15 (3), e0009303.

Pawlowski, Z.S., 2002. *Taenia solium*: Basic biology and Transmission. In: Singh, G., Prabhakar, S. (Eds.), *Taenia solium* cysticercosis: from basic to clinical science. CABI, Wallingford, pp. 1–13. https://doi.org/10.1079/9780851996288.0001.

Proctor, W.R., Bourdet, D.L., Thakker, D.R., 2008. Mechanisms underlying saturable intestinal absorption of metformin. Drug Metab. Dispos. 36 (8), 1650–1658.

Robinson, P., White, A.C., Lewis, D.E., Thornby, J., David, E., Weinstock, J., 2002. Sequential expression of the neuropeptides substance P and somastostatin in granulomas associated with murine cysticercosis. Infect. Immun. 70 (8), 4534–4538.

Rotermund, C., Machetanz, G., Fitzgerald, J.C., 2018. The therapeutic potential of metformin in neurodegenerative diseases. Front. Endocrinol. (Lausanne) 9, 400.

Shi, P., Liu, W., Tala, Wang, H., Li, F., Zhang, H., Wu, Y., Kong, Y., Zhou, Z., Wang, C., Chen, W., Liu, R., Chen, C., 2017. Metformin suppresses triple-negative breast cancer stem cells by targeting KLF5 for degradation. Cell Discov. 3 (1), 17010.

Spiliotis, M., Kroner, A., Brehm, K., 2003. Identification, molecular characterization and expression of the gene encoding the epidermal growth factor receptor orthologue from the fox-tapeworm *Echinococcus multilocularis*. Gene 323, 57–65.

Steelman, L.S., Abrams, S.L., Whelan, J., Bertrand, F.E., Ludwig, D.E., Bäsecke, J., Libra, M., Stivala, F., Milella, M., Tafuri, A., Lunghi, P., Bonati, A., Martelli, A.M., McCubrey, J.A., 2008. Contributions of the Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways to leukemia. Leukemia 22 (4), 686–707.

Sun, X.J., Crimmins, D.L., Myers, M.G., Miralpeix, M., White, M.F., 1993. Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1. Mol Cell Bio. 13 (12), 7418–7428.

Trelles, J.O., Roca, E., Ravens, R., 1952. Estudios sobre neurocisticercosis. I. Sobre la fina estructura de la membrana vesicular quística y racemosa –Deducciones patológicas. Rev. Neuro Psiquiatría. 15, 1–35.

Tucker, G.T., Casey, C., Phillips, P.J., Connor, H., Ward, J.D., Woods, H.F., 1981. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br. J. Clin. Pharmacol. 12 (2), 235–246.

Vazifehkhah, S., Ali, M.K., Babae, J.F., Hashemi, P., Alireza, M.S., Nikbakht, F., 2020. Evaluation of the ameliorative effects of oral administration of metformin on epileptogenesis in the temporal lobe epilepsy model in rats. Life Sci. 257, 118066.

Wang, L., Liang, P., Wei, Y., Zhang, S., Guo, A., Liu, G., Ehsan, M., Liu, M., Luo, X., 2020. *Taenia solium* insulin receptors: promising candidates for cisticercosis treatment and prevention. Acta Trop. 209, 105552.

Wang, S., Luo, X., Zhang, S., Yin, C., Dou, Y., Cai, X., 2014. Identification of putative insulin-like peptides and components of insulin signaling pathways in parasitic platyhelminths by the use of genome-wide screening. FEBS J. 281 (3), 877–893.

Xiong, Z.S., Gong, S.F., Si, W., Jiang, T., Li, Q.L., Wang, T.J., Wang, W.J., Wu, R.Y., Jiang, K., 2019. Effect of metformin on cell proliferation, apoptosis, migration and invasion in A172 glioma cells and its mechanisms. Mol. Med. Rep. 20 (2), 887–894.

Zhang, W., Liu, H.T., 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res. 12 (1), 9–18.

Zi, F., Zi, H., Li, Y., He, J., Shi, Q., Cai, Z., 2018. Metformin and cancer: An existing drug for cancer prevention and therapy. Oncol. Lett. 15 (1), 683–690.