1   Toxoplasmicidal *in vitro* effect of dehydroepiandrosterone on Toxoplasma gondii

2   extracellular tachizoytes

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22   Running Head: Toxoplasmicide *in vitro* effect of DHEA

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Abstract

53   Toxoplasmosis is a zoonotic disease caused by the apicomplexa protozoan parasite

54   *Toxoplasma gondii*. This disease is a health burden, mainly in pregnant women and

55   immunocompromised individuals, in whom they can cause death. Despite advances in

56   the medical area, nowadays there are no new drugs to treat toxoplasmosis. The standard

57   therapy to toxoplasmosis has not had progress for last seven decades; it is a combination

58   of sulfadiazine-pyrimethamine (S-P); which is co-administered with folic acid due to

59   the adverse effects of the drug. Several studies have shown that the conventional

60   treatment has limited effectiveness and severe adverse effects. Thus, the search of better

61   treatments with greater efficacy and without the adverse effects becomes relevant. In the

62   current work we demonstrate for the first time the parasiticidal effect of

63   dehydroepiandrosterone (DHEA), a steroid hormone produced by many mammals, on

64   extracellular tachyzoites (the infective stage of *T. gondii*). In vitro treatment with DHEA

65   reduces the viability of extracellular tachyzoites, and both the active and passive

66   invasion processes. The ultrastructural analysis of treated parasites showed that DHEA

67   alters the cytoskeleton structures, leading in the lost of the organelle structure and

68   organization, as well as, the lost of the cellular shape. On a molecular level, we

69   observed an important reduction of the expression of several proteins that are essential

70   for the motility and virulence of parasites when they were exposed to DHEA. These

71   results suggest that DHEA could be used as an alternative treatment against

72   toxoplasmosis.

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Introduction

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78 Toxoplasmosis is a zoonosis caused by the apicomplexa protozoan parasite,

79 *Toxoplasma gondii*, which is able to infect all warm-blooded animals [1-2]. This is a

80 worldwide disease with a prevalence average of 40 % [3]. Particularly, in Mexico the

81 sero-prevalence goes from 40-70% depending on the region of the country [4-5]. *T*

82 *gondii* infection can induce abortion, encephalitis, and in extremely cases, death. It is

83 considered a major opportunistic pathogen in patients with AIDS [6-7].

84 Human toxoplasmosis presents two phases: the acute and the chronic. In the acute

85 phase, parasite disseminate in the tachyzoite stage, the highly invasive and motile

86 asexual form. In this stage, parasite is able to cross any biological barrier, included the

87 placenta or the blood-brain barrier [8-11]. If the host is immunocompetent tachyzoites

88 will eventually differentiate into bradyzoites, the low replication form, and will begin

89 the tissue cyst formation [12]; this event defines the chronic infection, since tissue cysts

90 can stay forever in the host without provoking any apparent pathology [13].

91 Tachyzoite stage has a characteristic half-moon shape and an approximate size of 5 to

92 10 μm [14], as all members of Apicomplexa family; its motility depends on actomyosin

93 machinery that underlies the plasma membrane called glideosome [15]. *Toxoplasma*

94 counts with three specialized secretory organelles with particular proteins, which are

95 secreted in a controlled and specific manner during biological process, the micronemes

96 (MIC protein), rhoptries (ROP proteins) and dense granules (GRA proteins) [16].

97 *Toxoplasma* tachyzoite can carry out two types of invasion, active or passive. In the

98 active invasion *Toxoplasma* is the effector cell, and is the most important process due to

99 at majorly of the cell in the individuals are infected by mean this process. Firstly, the

100 tachyzoite must adhere to plasma membrane of the host cell; then glides propelled by

101 the glideosome that links to the host cell membrane via MIC2/MAP2 complex. It has

102   been described that parasite recognizes an unknown ligand of the host by its GPI-

103   anchored surface antigens, known as SAGs. Then, micorneme protein AMA1 and RON

104   proteins (RON2, RON4, RON5 and RON8) are secreted and a fusion of both plasma

105   membranes, called moving junction (MJ) is established [13, 17-18]. Parasite twirls

106   inside of the host cell at the same time that the PV is formed by the secretion of ROP

107   and GRA proteins. Inside of the non-fusogenic PV, the parasite is replicated by

108   endodiogeny, an asexual replication form, that from the boundaries of a mature mother

109   parasite forms two daughters' cells [19-20].

110   Passive invasion occurs in all phagocytic cells, these being the effector cells for the

111   process. First, the parasite adheres to the plasma membrane of a phagocytic activated

112   cell, surrounded by the plasmatic membrane elongations and internalized towards the

113   cytoplasm in a phagocytic vacuole [13]. Once inside, the parasite evades the immune

114   response transforming the phagocytic vacuole in to a parasitophorous vacuole (PV) via

115   the phosphorylation of the host Immune-Related GTPases (IRGs) via a complex that

116   includes ROP and GRA proteins. This prevents their oligomerization and recruitment to

117   the PVM leading in the inhibition of the vacuole lysis and parasite clearance by

118   macrophages then parasite is able to replicate [13, 21-23].

119   Conventional therapy against toxoplasmosis consists of a mixture of sulfadiazine-

120   pyrimethamine that was established in the 50's decade. Since then, minor advances have

121   been made in the treatment of the zoonosis [24-26]. Although sulfadiazine –

122   pyrimethamine are synergic it is known that they present severe side effects. Since

123   pyrimetamine is a folic acid antagonist it has been associated with bone marrow toxicity

124   while sulfadiazine causes hypersensitivity and allergic reactions up to 20% of

125   population [27-28]. Besides than this conventional treatment has a limited effectiveness,

126   mainly on chronic stage disease, there is not available vaccine for human use.

127   DHEA is a steroid hormone that is produced, from cholesterol, in the adrenal glands,

128   gonads and brain, and is synthesized from pregnenolone by the action of the 17, 20-

129   desmolase enzyme [29]. It is the most abundant hormone circulating in mammals and

130   can also be a precursor of sexual steroids [30]. The sulphated form of DHEA is majorly

131   found in blood circulation and the free DHEA form (the active form) is only the 3-5 %

132   of the total concentration. Although DHEA is a hormone produced by the organism, it

133   has been postulated for its therapeutic usage as a parasiticide agent. In vitro, low

134   concentrations of DHEA inhibit proliferation, adhesion and motility of *Entamoeba*

135   *histolytica* trophozoites, while high concentrations induce the lysis of the parasite [31].

136   DHEA reduces 75% the reproduction of *Taenia crassiceps* cysticercus, in vitro; and in

137   murine model infected with metacestodos of *Taenia* the parasitic charge was 50%

138   reduced when mice were previously treated with the hormone [32]. In a toxoplasmosis

139   acute infection model, DHEA was administrated, pre and pos-infection to

140   immunosupressed mice; DHEA reduced mortality in a 65 % in the pre-treated mice and

141   in a 50 % in post-treated mice; besides this treatment reduced the number of brain cysts

142   in pre-treated infected mice in 90 % and in post-treated mice in 60 % when compared to

143   the control [33]. The effect of estradiol and progesterone has been studied in

144   extracellular tachyzoites of *T. gondii*, showing that estradiol exposure increases the

145   intracellular calcium concentration via the acidic organelles, which increases the

146   secretion of MIC2 and gliding motility in consequence. Progesterone exposure increases

147   the intracellular calcium concentration via the neutral organelles, presenting a contrary

148   effect than observed with estradiol exposure. Although these hormones are able to

149   trigger the calcium signalling in *T. gondii* tachyzoites, none receptor has been reported

150   so far [34].

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151   The development and research of new drugs against toxoplasmosis is relevant; in the

152   search of new therapies with practical application, our research group has studied the

153   effect of sex steroid hormones on the immune response to different parasitic infections.

154   In the present work, we assessed the effect of DHEA, alone or in combination with the

155   conventional treatment S-P, on *Toxoplasma* extracellular tachyzoites. Our results

156   suggest that DHEA could be recognized by a cytochrome b5 family heme/steroid

157   binding domain-containing protein inducing a reduction of passive and active invasion

158   by the modulation of the expression of proteins that are essential during the invasion

159   process, as well as some virulence factors.

Results

**The treatment with DHEA decreases the viability of *Toxoplasma gondii* extracellular tachyzoites.**

In order to known the effect of dehydroepiandrosterone hormone on the viability of *Toxoplasma* we exposed extracellular tachyzoites to increasing DHEA concentrations for 30 minutes and two hours. In viability assay, all micromolar concentrations used induced a considerable decrease in the parasite in both times tested. At 30 min, a decrease of between the 25 to 40 % was observed for the 1, 10, 50, 80, 100, 200 and 400 μM concentrations and the maximum effect was observed with 600 μM of DHEA, which reduced the viability in approximately 55% (Fig 1A, grey bars). At two hours, a decrease of the viability of 45 % was observed for 1 and 200 μM concentrations, and the maximum effect was observed for the 10, 100, 400 and 600 μM concentrations that reduced viability in approximately 58 to 62 % (Fig 1A, white bars). These results suggest that the viability of extracellular tachyzoites of *Toxoplasma* is compromised when they are exposed to therapeutic concentrations of DHEA.

The combined treatment with DHEA / S-P presents a greater diminution of the viability of *Toxoplasma gondii* extracellular tachyzoites than the individual effect of either treatment.

There are the possibility of DHEA could be used as an auxiliary compound in the treatment against Toxoplasma infection, we tested the effect of the conventional treatment with S-P combined with DHEA. First, we used equal concentrations of both drugs at 30 minutes and two hours (Fig 1B). We observed a considerable diminution of the viability with 200 μM concentration in both times tested. At 30 minutes, a decrease between the 40 to the 50 % of the viability was observed since 200, to 600 μM concentrations (Fig 1B, grey bars). The effect observed at 200 μM resulted

185   approximately 10 % higher than the effect observed at the same concentration in the S-P

186   treatment (Figs 1B and 1C, grey bars). In contrast, at two hours the parasite viability

187   was reduced approximately 40 % with the same concentration (Fig 1B, white bars); this

188   reduction are in concordance with reduction induced by DHEA alone; however, the S-P

189   treatment have a better effect inducing a 20 % viability decreased (Figs 1A and 1c,

190   white bars). All the other tested concentrations showed a similar effect (Fig 1B, white

191   bars).

192   Parasites were also treated with a constant concentration of 10 μM of DHEA combined

193   with 600 or 800 μM of S-P for 30 minutes and two hours. At 30 minutes, both

194   combinations of concentrations showed a reduction of the viability of approximately 35

195   % (Fig 1B, grey bars); this effect is similar for 10 μM DHEA alone treatment. However,

196   the conventional treatment (S-P) showed a better effect on viability that these modality

197   of combination at 30 min (10 μM DHEA/600 and 800 μM S-P) (Fig 1A vs 1C, grey

198   bars). At two hours, the effect for both combinations of concentrations is lower than

199   individual treatments (Fig 1B vs 1C, white bars).

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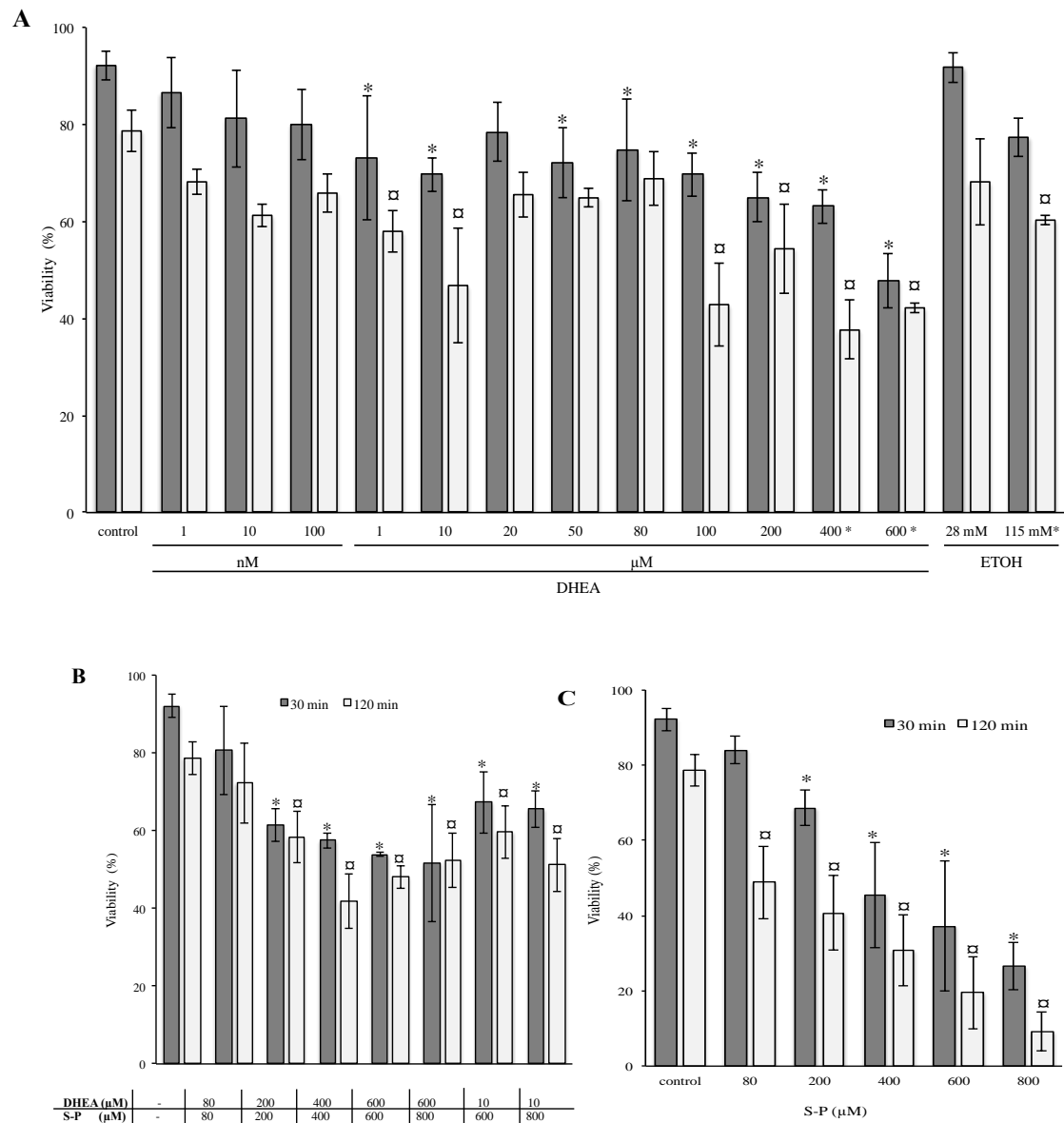
Fig 1. Effect of DHEA on *T. gondii* extracellular tachyzoites viability. A) DHEA B)

DHEA/S-P and C) S-P treatment, in X axis showed final concentration of each drug; in

Y axis = percentage of viability. Grey bars indicate 30 min and white bars correspond to

2 h of treatment. Control, tachyzoites without treatment in PBS; ETOH correspond to

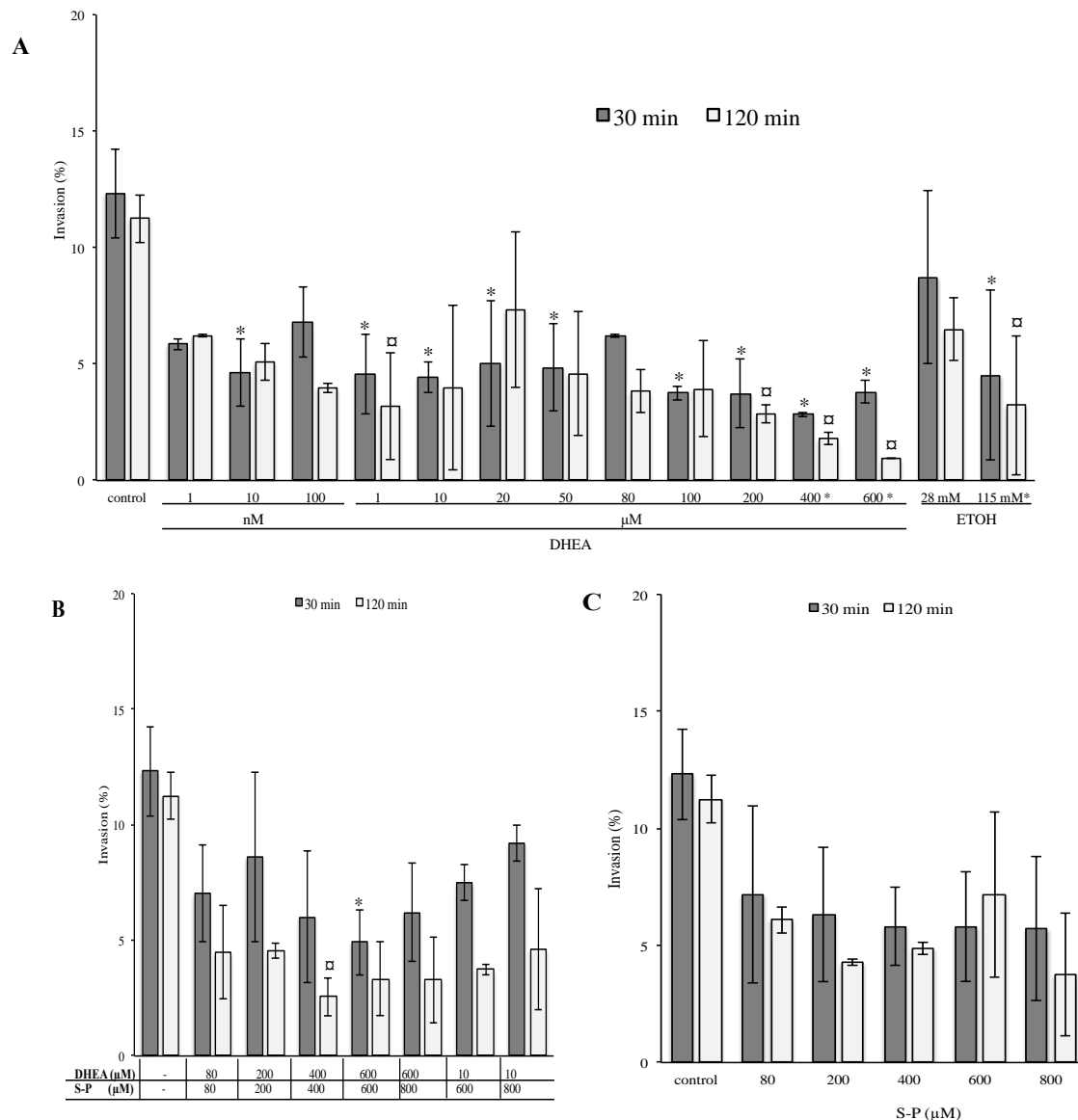DHEA solution vehicle (ethanol 2.8 and 11.5 μL / 100 μL; * in Y axis, indicate the

concentration correspond to high quantity of ethanol used). (*, ¤) Statistical significance

compared to the control according to exposure time. P<0.0001

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209 **The treatment with DHEA and DHEA/S-P reduces the active invasion process.**

210 After to treat the tachyzoites with DHEA and DHEA/S-P at several concentrations, we

211 analyse if these parasites were capable to penetrate their human host cell. Hep-2 cells

212 monolayers were exposed at pretreated tachyzoites during 30 min or 2 h (Fig 2). At 30

213 min, the tachyzoite invasion capacity was inhibit in around 60% respect to the control

214 without treatment, in almost all DHEA concentrations used (asterisks, Fig 2A, grey

215 bars). While, tachyzoites pretreated for 2 h, exhibited a 70% approximately of decrease

216 in invasion process when they were treated with 1, 200 or 400 μM of DHEA, respect to

217 the control without treatment. The maximum effect was observed with 600 μM DHEA,

218 reducing the active invasion in approximately 90 % vs tachyzoites without treatment

219 (Fig 2A, white bars). While DHEA/S-P treatment, only presented significant

220 differences, a decrease of around 50% at 600 μM of both drugs for 30 min (Fig 2B, grey

221 bars); and at 400 μM DHEA/S-P for 2 h, reach approximately a diminution of the active

222 invasion 75 % respect to the parasites without treatment (Fig 2B, white bars). The

223 treatment of the extracellular tachyzoites with S-P had not significant differences in the

224 active invasion independently the concentrations and times tested (Fig 2C).

11

Fig 2. Effect of DHEA in the active invasion process. A) DHEA B) DHEA/S-P and

C) S-P treatment, X axis = final concentration of each drug; Y axis = percentage of

HEp-2 cells that contained at least one parasitophorous vacuole in the cellular

cytoplasm. Grey bars, 30 min and white bars, 2 h of treatment; control, PBS ETOH

correspond to DHEA solution vehicle (ethanol 2.8 and 11.5 µL / 100 µL; * in Y axis,

indicate the concentration correspond to high quantity of ethanol used). (*, ¤) Statistical

significance compared to the control according to exposure time. P<0.0001

235   **The treatment with DHEA reduces the passive invasion process.**

236   *T. gondii* have the capacity to invaded all nucleated cells, to introduce at phagocytic

237   cells like macrophages, it used the active machinery of the phagocytic cells themselves,

238   this kind of invasion is called passive. In order to know if DHEA has an effect in this

239   process we put together fresh macrophages with pretreated extracellular tachyzoites for

240   30 min or 2 h (Fig 3). First, we compared the percentage of phagocytized untreated

241   tachyzoites by LPS-activated macrophages against the percentage of phagocytized

242   untreated tachyzoites by non-activated macrophages. Activated macrophages

243   phagocytized approximately 40% more untreated tachyzoites compared with non-

244   activated macrophages (Fig 3A, activated vs non-activated). For this reason we use

245   activated macrophages for the consecutive assays. Passive invasion was reduced in

246   approximately 35 % when activated macrophages were exposed to pretreated

247   tachyzoites with 600 μM DHEA for 30 min (Fig 3A, grey bars), and to pretreated

248   tachyzoites with 80, 400 and 600 μM DHEA for 2 h (Fig 3A, white bars). The

249   combined (DHEA/S-P) and the conventional (S-P) treatments on extracellular

250   tachyzoites have no effect in the passive invasion, independently of the concentrations

251   and times (Fig 3B and 3C, respectively).
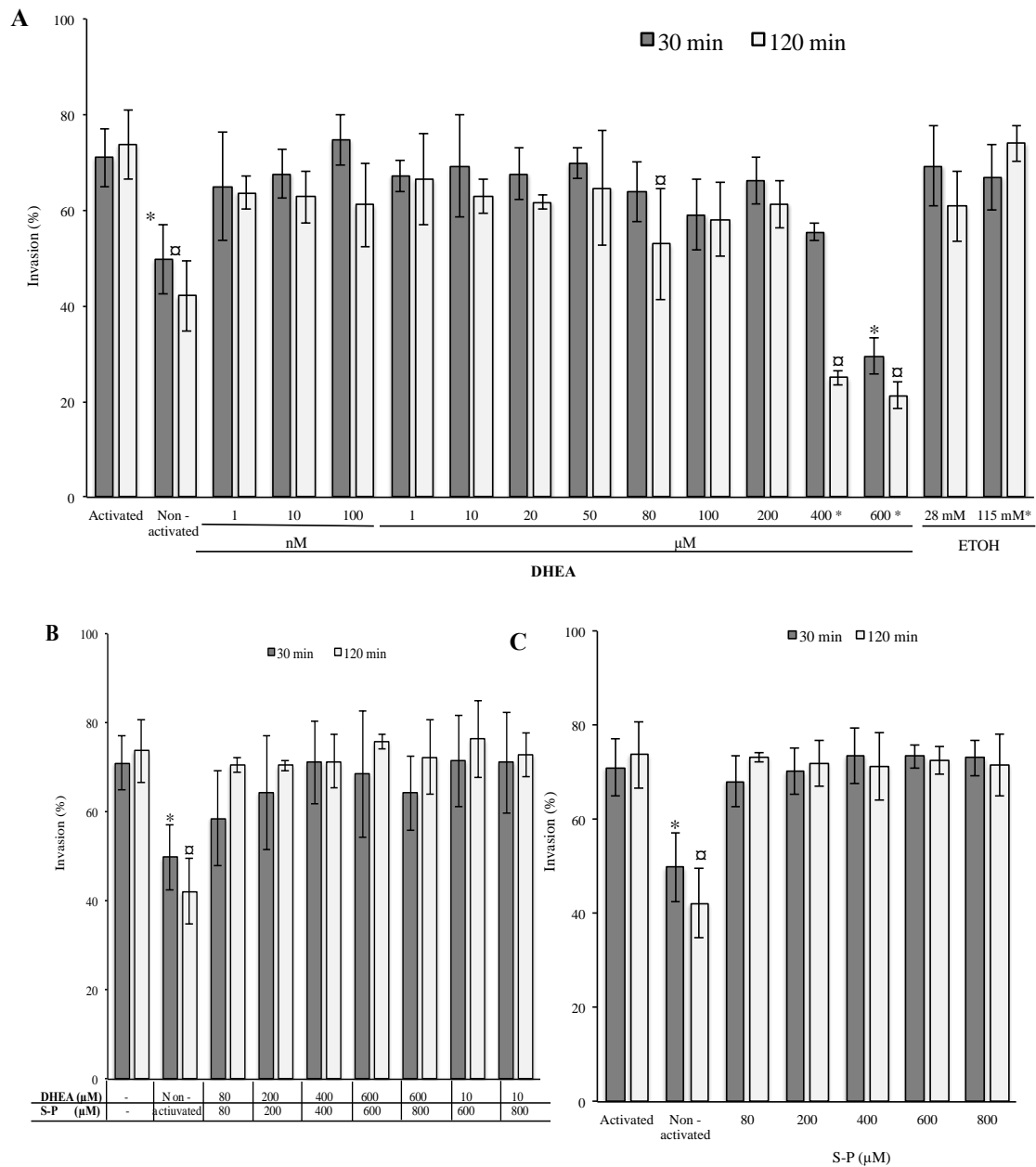
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Fig 3. Effect of DHEA in the passive invasion process. A) DHEA B) DHEA/S-P and

C) S-P treatment, X axis = final concentration of each drug; Y axis = percentage of

macrophages that contained at least one PV in the cellular cytoplasm. Grey bars, 30 min

and white bars, 2 h of treatment; control, PBS ETOH correspond to DHEA solution

vehicle (ethanol 2.8 and 11.5 μL / 100 μL; * in Y axis, indicate the concentration

correspond to high quantity of ethanol used). (*, ¤) Statistical significance compared to

the control according to exposure time. P<0.0001

261   **Tachyzoites treated with DHEA and DHEA / S-P present morphological changes.**

262   We analysed if the decrement in invasion process could be related to morphological

263   changes induced by the DHEA treatment on extracellular tachyzoites. The ultrastructure

264   images of extracellular parasites treated as in the viability assay, for all concentrations

265   of each treatment, DHEA and S-P alone and DHEA/S-P, were obtained by TEM (Figs 4

266   and 5). The untreated and vehicle control (ethanol) tachyzoites, are showed in Fig 4A

267   and 4B, respectively. The DHEA treatment at 10 μM – 30 min, preserves all the typical

268   structures such as micronemes, rhoptries, dense granules, nuclei and mitochondria.

269   Interestingly, we were capable to observe the presence of granules apparently of

270   amylopectin, which are exclusive of bradyzoite stage; and some areas of plasmatic

271   membrane look wavy (Fig 4C). Importantly, the effect of DHEA on tachyzoites

272   structure is related to the concentration and time used. At 100 μM DHEA for 30 min,

273   the parasites seem to lose their typical half-moon shape and some of them present

274   amylopectin granules (Fig 4E). While with 400 μM DHEA (30 min), parasites look a

275   little swollen and present bigger amylopectin granules (Fig 4G). At 30 minutes with 600

276   μM DHEA, some parasites seem to preserve their half-moon shape, with a

277   reorganization of the organelles and the presence of amylopectin granules; however, at

278   this concentration of DHEA there are a greater amount of phantom structures in the

279   samples (Fig 4I). Longer time of DHEA exposition induced greater changes in the

280   extracellular tachyzoites morphology. Parasites exposure to 10 and 100 μM of DHEA

281   by two hours, showed an amoeboid shape with a totally lost of the intracellular

282   organization and the apical polarity; the dense granules lost their circle shape and the

283   presence of some amylopectin granules were observe to (Fig 4D and 4F). In the same

284   time but exposure with 400 and 600 μM of DHEA, parasites look inflated with a

15

285   balloon shape, although they seem to preserve organelles typical organization, as well as

286   the apical polarity (Fig 4H and 4J).
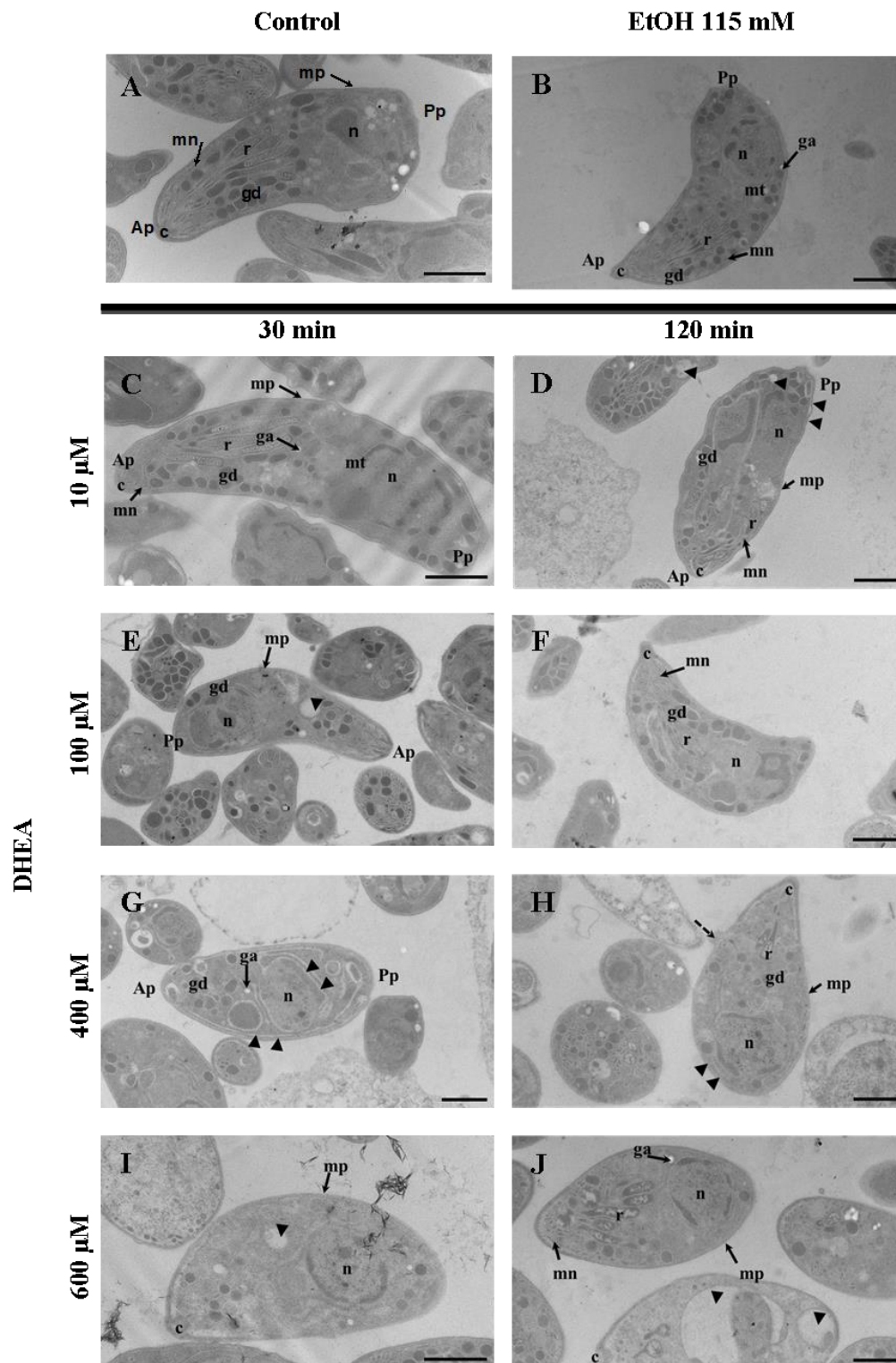
Fig 4. Effect of DHEA in the ultrastructure of *Toxoplasma gondii* extracellular

tachyzoites. A) Morphology of extracellular tachyzoites untreated and B) tachyzoites

290 exposure to ethanol, corresponding at 2h of exposure. Extracellular tachyzoites

291 exposure to C) 10 µM, E) 100 µM, G) 400 µM and I) 600 µM final concentration of

292 DHEA for 30 min; or D) 10 µM, F) 100 µM, H) 400 µM and J) 600 µM final

293 concentration of DHEA for 2 h.. c, conoid; r, rhoptries; mn, micronemes; gd, dense

294 granules; ga, amylopectin granules; n, nuclei; m, plasma membrane; re, endoplasmic

295 reticulum; mt, microtubules; Ap, apical pole; Pp, posterior pole Bars= 1 µm.

296

297 In the combined treatment, DHEA/S-P at 10/800 µM, 600/600 µM and 600/800 µM, the

298 effect was observed since 30 min of exposure and it was consistent after 2 h (Fig 5). At

299 10/800 µM DHEA/S-P the parasites lose their typical shape and present amoeboid and

300 elongated shape, they also start to lose the intracellular organization and present

301 amylopectin granules (Fig 5A-B). At higher concentrations of DHEA/S-P 600/600 µM,

302 some parasites preserved the typical shape lose the organization of the organelles,

303 finding the rhoptries at the posterior pole, the dense granules lost their circled shape,

304 and they present amylopectin granules (Fig 5C-D). Finally, with 800/800 µM of

305 combined treatment, the appearance of tachyzoites was amorphous with loss of the

306 organelles organization and again, presence of amylopectin granules (Fig 5E-F)

307 The conventional treatment induced important changes in the extracellular tachyzoites,

308 independently of concentration or time exposure. At 200 and 800 µM of S-P for 30 min

309 and 2 h, the tachyzoites loose their typical shape and was possible find tachyzoites with

310 amoeboid, elongated or amorphous shape, they present amylopectin granules, loose the

311 apical polarity and the organization of the organelles (Fig 5G-J).
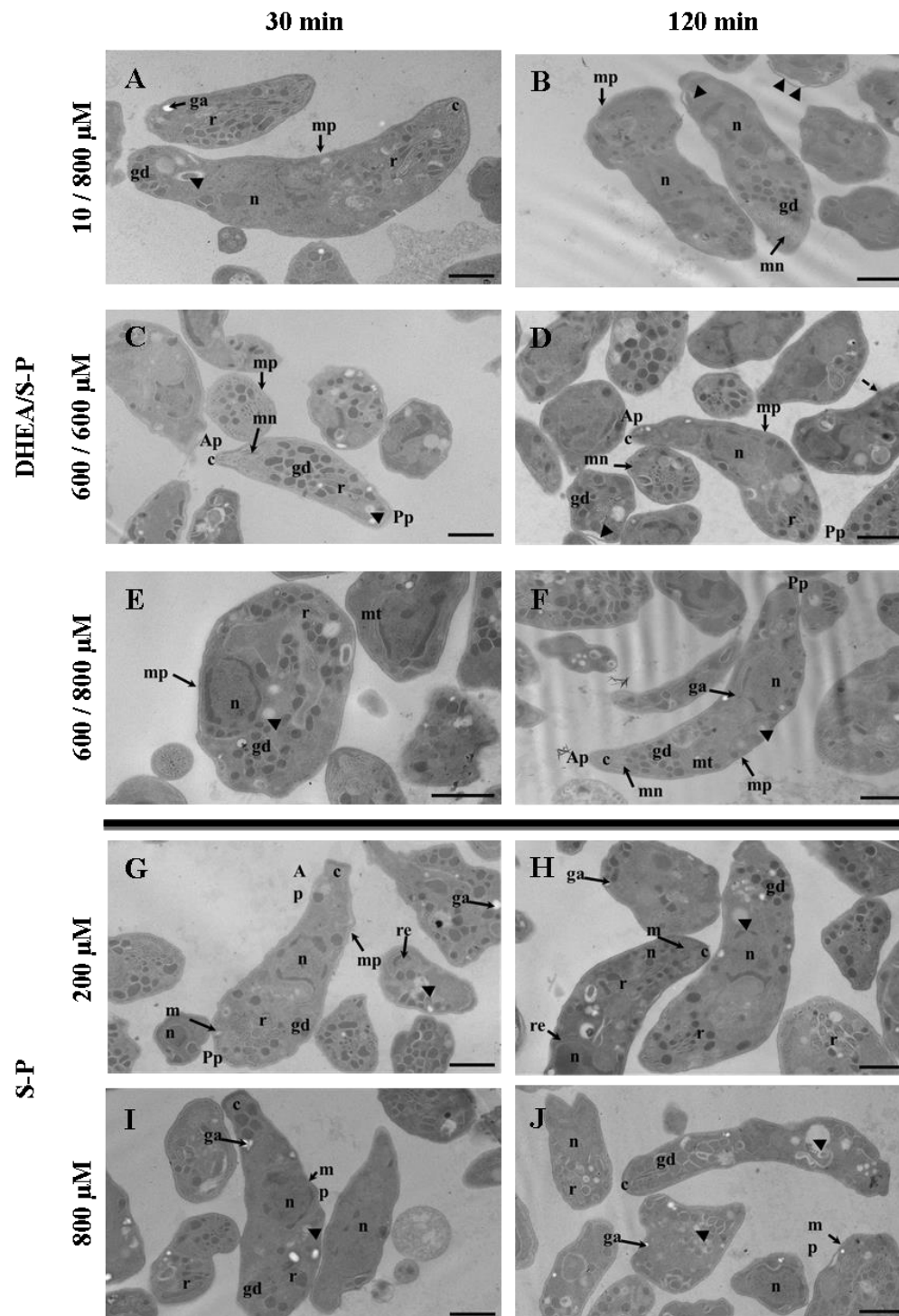
Fig 5. Effect of DHEA / S-P in the ultrastructure of *Toxoplasma gondii* extracellular tachyzoites. Extracellular tachyzoites treated with A) 10 / 800 μM, B) 600 / 600 μM, C) 600 / 800 μM final concentration of DHEA / S-P, respectively for 30 minutes or D) 10 / 800 μM, E) 600 / 600 μM, F) 600 / 800 μM final concentration of DHEA / S-P, respectively for 120 minutes. c, conoid; r, rhoptries; mn, micronemes; gd,

318   dense granules; ga, amylopectin granules; n, nuclei; m, plasma membrane; mt,

319   microtubules; Ap, apical pole; Pp, posterior pole. Bar = 1 µm.

320   **Treatment with DHEA, S-P or DHEA / S-P induces changes in the protein**

321   **expression since 30 minutes of exposure.**

322   To determine the effects that the treatments with DHEA, S-P and DHEA / S-P have at

323   molecular level, we used in extracellular tachyzoites to compare the protein profile of

324   the treated parasites against the protein profile of tachyzoites without treatment (Fig 6).

325   The protein profile of the control exhibited 159 spots (Fig 6A); the group treated with

326   DHEA 10 µM for 30 min exhibited 165 spots, which 105 were match with control (Fig

327   6B); the group treated with S-P 800 µM for 30 min exhibited 126 spots, which 99 were

328   match with control (Fig 6C); while the group treated with DHEA / S-P 10 / 800 µM for

329   30 min exhibited 213 spots, which 113 were match with control (Fig 6D). Protein

330   profiles were analyzed as described in methods and materials section, and we selected

331   the proteins that showed greater changes in their expression between the treatments and

332   respect to the control. Thirty proteins were identified by its molecular weight and

333   isoelectric point (Table 1), as described in methods and materials section, and were

334   classified by their probable location (Fig 7A). We observed that most of the proteins

335   that change their expression are dense granules proteins, followed by proteins from

336   plasma membrane and cytoplasm (Fig 7A). Then, we graphed the proteins that

337   diminishes their expression respect to the control (Fig 7B), and the proteins that

338   maintain or increase their expression respect to the control (Fig 7C). We can observe

339   that treatment with DHEA leads in a diminution of the expression of proteins from

340   dense granules, micronemes, apicoplast, peroxisome, plasma membrane, mitochondria

341   and cytoskeleton; while proteins from cytoplasm increases their expression (Fig 7 insets

342   B and C, light grey bars). S-P treatment provokes the reduction in the expression of

343   proteins from dense granules, micronemes, cytoplasm, apicoplast, peroxisome, plasma

344   membrane and mitochondria; and the increment of proteins from rhoptries, IMC and

345   cytoskeleton (Fig 7 insets B and C, dark grey bars). Combined treatment with DHEA /

346   S-P, induces a lower expression of proteins from dense granules, micronemes,

347   cytoplasm, apicoplast and peroxisome; and a higher expression of proteins from

348   rhoptries, plasma membrane and mitochondria (Fig 7 insets B and C, black bars).
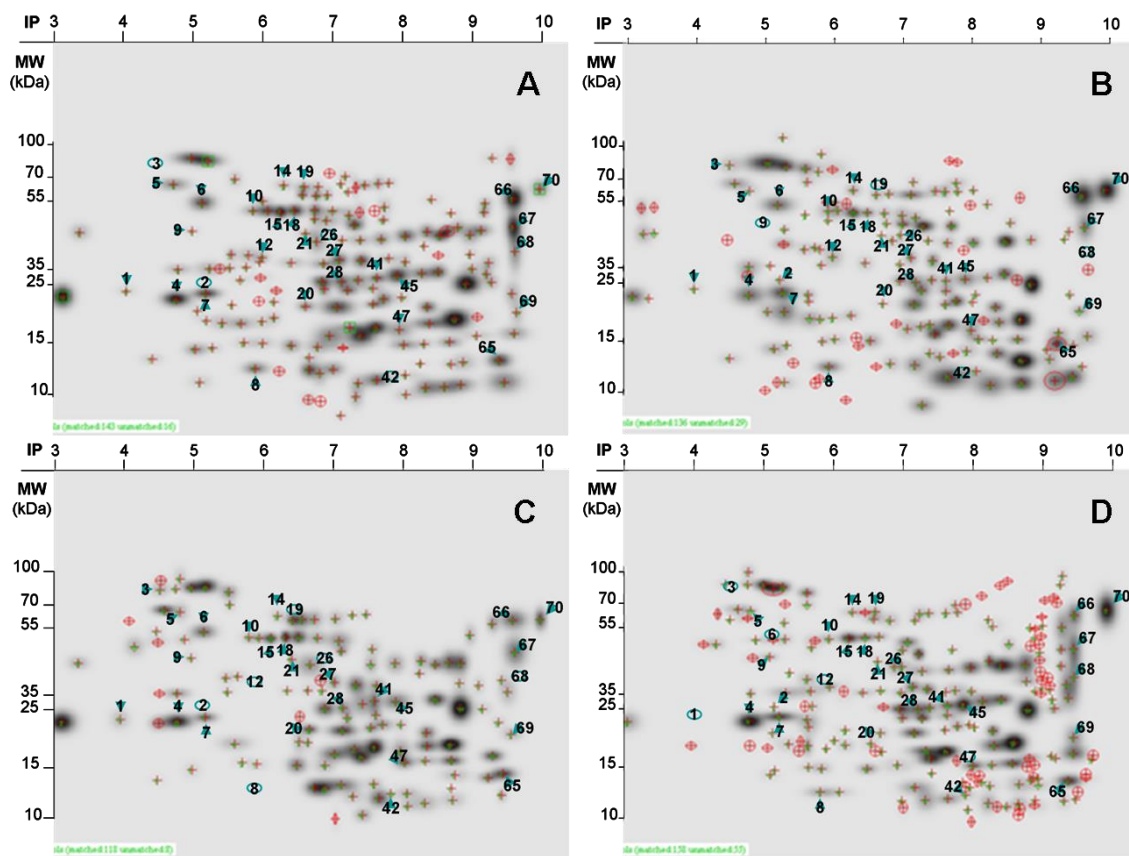
Fig 6. Proteomic profile of whole extract of extracellular tachyzoites. 30 minutes of treatment with: A) Control without treatment; B) DHEA 10 μM; C) S-P 800 μM and D) DHEA / S-P 10 / 800 μM. Arrowheads point out the spots (identified in table 1) that were identified in all treatments; the spots that are absent in treatments respect to the control are circled.

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Table 1. Protein differential expression in whole extract of extracellular tachyzoites									
						Relative expression compared to the control			
Spot number	Protein name	Uniprot KB ID	Theoretical Mr / IP	Experimental Mr / IP	Possible location	Ctrl	DHEA (10 µM)	S-P (800 µM)	DHEA /S-P (10/800 µM)
1	Dense granule protein (GRA1)	P13403	17.85 / 4.13	23 / 4.1	Dense granules	*	*	**	_
2	Cytochrome b5 family heme/steroid binding domain-containing protein	A0A139XPA0	26.25 / 5.18	27 / 5.1	Unknown	_	*	_	*
3	Microneme protein (MIC2)	O00816	82.61 / 4.45	84 / 4.5	Micronemes	_	**	*	_
4	Dense granule protein (GRA7)	O00933	23.23 / 4.95	22.9 / 4.9	Dense granules	***	**	***	****
5	Microneme protein (MIC4)	Q9XZH7	63.02 / 4.84	63 / 4.66	Micronemes	***	*	**	*
6	Micronemal protein (MIC1)	O00834	46.97 / 5.20	52.7 / 5.15	Micronemes	***	**	*	_
7	Dense granule protein (GRA6)	Q27003	24.02 / 5.47	23.5 / 5.35	Dense granules	***	**	*	***
8	Dense granule protein (GRA5)	Q07828	12.97 / 5.81	12.5 / 5.98	Dense granules	***	***	_	**
9	Rhoptry protein (ROP1)	A0A125YP48	47.99 / 4.9	45 / 4.99	Rhoptries	*	_	**	**
10	Enolase 2	Q9BPL7	48. 29 / 5.67	53 / 5.71	Cytoplasm	**	**	*	*
12	Inosine-5'-monophosphate dehydrogenase	Q4VRV8	40.36 / 6.08	38.5 / 6.01	Cytoplasm	*	**	_	_
14	Rhoptry neck protein (RON4)	B6KJ32	65.34 / 6.49	65.8 / 6.41	Rhoptries	*	**	*	**
15	Anamorsin homolog	B9Q0C2	47.56 / 6.49	53 / 6.38	Cytoplasm and mitochondria	****	***	**	****
18	Elongation factor Tu	Q9TMM9	44.31 / 6.52	52 / 6.5	Apicoplast	****	*	***	**
19	Peroxisomal catalase	Q9XZD5	57.27 / 6.71	63 / 6.69	Peroxisome	**	_	_	*
20	Profilin	A0A086PNN0	20.78 / 6.51	21 / 6.7	Cytoskeleton	**	*	***	*
21	Gliding associated protein (GAP40)	E0AE39	43.08 / 6.61	44 / 6.6	Inner membrane complex	**	*	**	*
26	Acid phosphatase GAP50	A0A086PXK7	46.60 / 6.95	46 / 6.97	Inner membrane complex	_	*	_	**
27	Dense granule protein (GRA4)	Q27002	34.08 / 7.19	36.5 / 7.12	Dense granules	***	*	*	**
28	Major surface antigen p30 (SAG1)	P13664	29.80 / 6.84	26 / 7.1	Plasma membrane	***	**	*	***
41	Rhomboid-like protease (ROM1)	Q695U0	32.83 / 7.69	30 / 7.68	Plasma membrane	***	**	***	**
42	Actin depolymerizing factor (ADF)	A0A086PI60	12.94 / 7.92	12.5 / 7.8	Cytoskeleton	_	_	*	*
45	Rhomboid-like protease (ROM3)	Q6IUY1	29.34 / 8.19	30 / 7.95	Plasma membrane	***	*	**	****
47	Dense granule protein (GRA2)	P13404	17.46 / 8.21	17 / 8.0	Dense granules	**	**	**	_
65	50S ribosomal protein L14	Q9XQQ6	14.08 / 9.77	13.9 / 9.4	Apicoplast	***	_	**	**
66	Rhomboid-like protease (ROM4)	Q695T8	69.66 / 9.24	65.5 / 9.51	Plasma membrane	****	***	**	**
67	Cytochrome b	O20672	41.59 / 9.25	46.5 / 9.51	Mitochondria	***	*	**	**
68	Phosphatidylserine decarboxylase proenzyme 1	Q1PCQ8	39.44 / 9.61	39 / 9.61	Mitochondria	**	_	_	**
69	Dense granule protein (GRA3)	B6KEU8	24.24 / 9.46	20 / 9.65	Dense granules	**	*	*	***

21

70	Diacylglycerol kinase catalytic domain-containing protein	A0A139XS45	69.58 / 10.02	63 / 9.97	Cytoplasm	*	***	**	****
Proteins were identified by molecular weight and isoelectric point using the program TagIdent in ExPASy web page (https://web.expasy.org/tagident/).									

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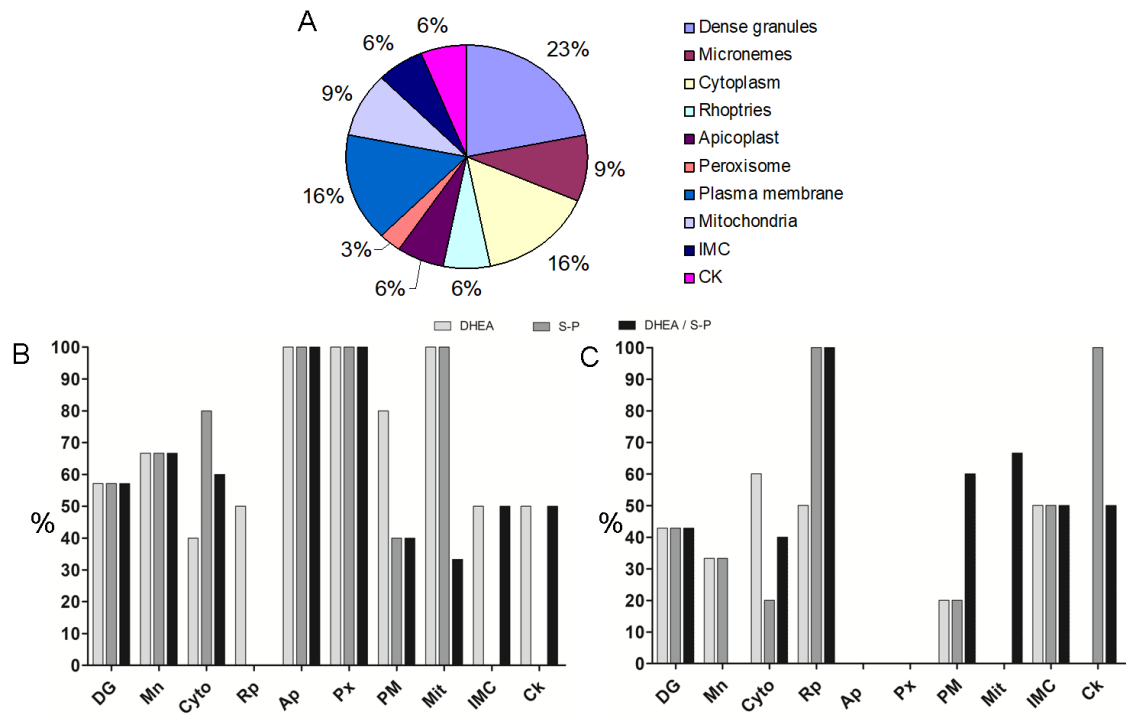
Fig 7. Classification of proteins that exhibited differential expression. A) Proteins that change their expression after 30 minutes of treatment with DHEA 10 μM, S-P 800 μM or DHEA / S-P 10 / 800 μM, grouped by their probable location. B) Percentage of proteins that decrease their expression respect to the control without treatment (y axis). C) Percentage of proteins that maintain or increase their expression respect to the control without treatment (y axis). X axis, probable location; DG, dense granules; Mn, micronemes; Cyto, cytoplasm; Rp, rhoptries; Ap, apicoplast; Px, peroxisome; PM, plasma membrane; Mit, mitochondria; IMC, inner membrane complex; Ck, cytoskeleton.

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370 **Interaction of DHEA with the Cytochrome b5 family heme/steroid binding**

371 **domain-containing protein**

372 Interestingly the spot number 2, which exhibited an experimental molecular weight of

373 27 KDa and isoelectric point of 5.1, and that is expressed only in the protein profile of

374 tachyzoites treated with DHEA; was identified as a cytochrome b5 family heme/steroid

375 binding domain-containing protein with a theoretical molecular weight of 26.25 KDa

376 and isoelectric point of 5.18. The primary sequence of the protein was aligned in the

377 NCBI web site with the BLAST program

378 (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearc

379 h&LINK_LOC=blasthome) and the protein was aligned with other steroid binding

380 proteins from other Eukaryotes (S1 figure A-C), then we aligned only the domain

381 section (aminoacids from 129 to 176) and the result was similar (S1 figure D-F).

382 The *T. gondii* model generated is the best that can be obtained given that the only

383 available template has a 36.9% homology; this corresponds to the 111 residues located

384 at the carboxyl-terminus of the full 243 residue protein. Most of the residues know to

385 interact with the heme group in the PGRMC1 structure are identical in our predicted

386 structure for *T. gondii* PGRMC. The resulting model has a heme group partially buried

387 and contributing significantly to the binding of all of the ligands tested (Fig 8). In every

388 case, the three best results for each ligand were in contact with the heme group on the

389 surface of the protein. Notably, progesterone is the most tightly bound ligand followed

390 by dehydroepiandrosterone, testosterone and 4-5 alpha dihydrotestosterone (Table 2).

391 Given that residue TYR158 (numbering based on the whole sequence cloned) provides

392 the fifth coordination to the heme-iron, forcing ligand interaction to occur on the

393 unoccupied side of the heme group.

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394
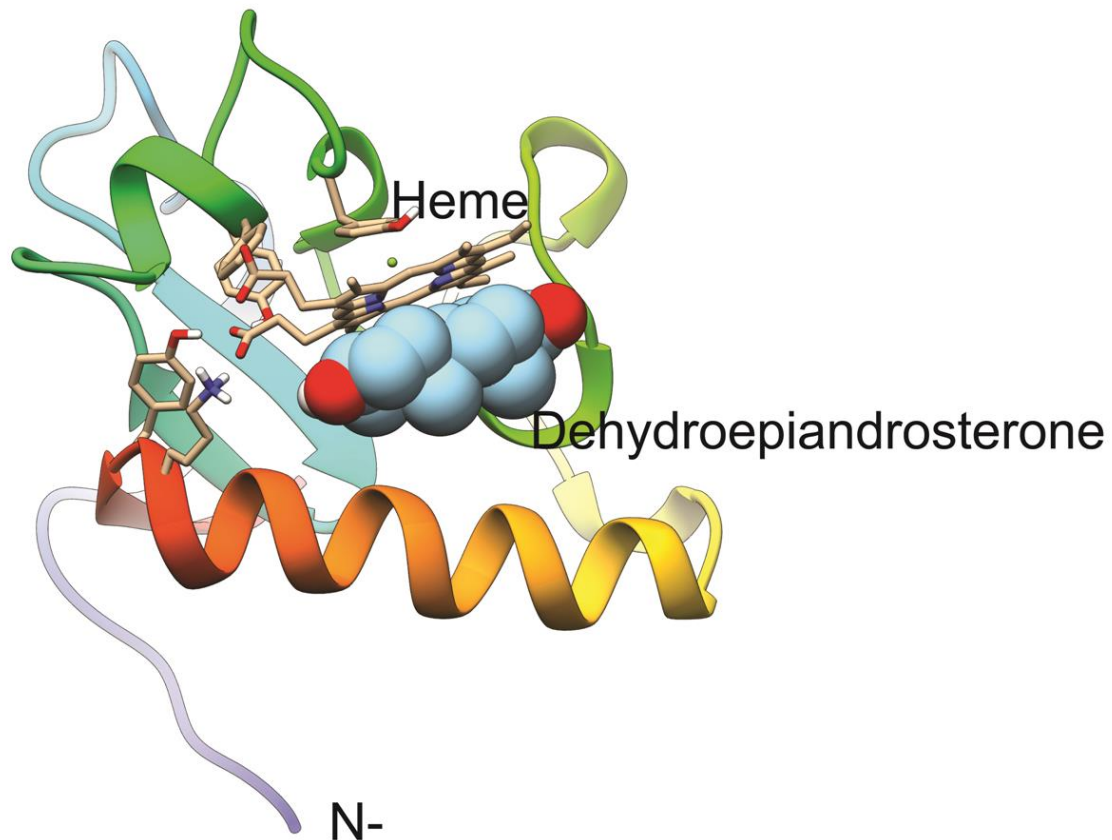
Fig 8. Model for *T. gondii* PGRMC homolog and its docking to DHEA. The model

for PGRMC contains a binding pocket for a heme group that functions as the binding

site for DHEA. TYR158 binds the heme group on one face while the other binds

DHEA, blocking any interaction at that site.

Table 2. Ligands that present best affinities to TgPGRMC	
Ligand	Vina kcal/mol
Octanoate	-4.1
Decanoate	-4.4
Dodecanoate	-4.6
Myristate	-5
Palmitate	-4.6
Stearic	-5.1
Oleate	-5.4
Linoleate	-5.5

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DHEA	*-7.4*
4-5 alpha-Dihydrotestosterone	*-7.4*
Aldosterone	-7.1
Estriol	-7.2
Cholesterol	-6.6
Progesterone	*-7.6*
Testosterone	*-7.4*
Corticosterone	-6.8
Beta-estradiol	-6.7
Cortisol	-6.5
Pyrimethamine	-5.9
Sulfadiazine	-5.5

399   Pyrimethamine and sulfadiazine were also found to bind the heme group but with

400   significantly lower affinity (bottom of Table 2). Given that these affinities are about 1.5

401   kcal/mol lower, it is likely that they are non-specific as well as is the case for fatty acids

402   included in the docking.

403   It is unknown if *T. gondii* PGRMC is able to dimerize as its template (*Homo sapiens*

404   PGRMC1), but the interactions with the ligands tested in the present work would block

405   or compete a similar interaction.

Discussion

406   DHEA induces a decrement in the viability of extracellular tachyzoites at 100 μM at

407

408   both times tested (30 min and 2 h); even if effect was lower than the observed with

409   standard therapy (S-P); results are similar to the obtained when trypomastigotes of *T.*

410   *cruzi*, another intracellular parasite were treated with 128 μM final concentration of

411   DHEA combined with melatonin for 24 h [35]. Different to the standard treatment,

412   whose response was directly proportional to the concentration, the effect obtained with

413   DHEA exposure was independent on the concentration. This suggests that both drugs

414   have different targets inside of the tachyzoite. In order to evaluate an accumulative

415   effect of the DHEA with S-P we tested the combined treatment, resulting in a similar

416   effect as the obtained when DHEA alone was administered. It is important to mention

417   that previously reported DHEA parasiticide effect depends on the administration

418   scheme, parasite lineage and experimental conditions.

419

420   Once we determined that viability was affected by DHEA treatment, we assessed if the

421   hormone has an effect in the invasion process, which is the most important biological

422   process for the establishment and maintenance of the infection; as tachyzoites are able

423   to infect phagocytic and non-phagocytic cells in two different mechanisms that has been

424   defined as passive and active invasion, respectively, we determined the effect of the

425   DHEA in both.

426   Tachyzoites reduced its ability to invade HEp-2 monolayers, when they were treated

427   with DHEA; this reduction was higher than the observed with the conventional

428   treatment with S-P. Differently to passive invasion, active invasion was reduced when

429   parasites were treated with the DHEA / S-P combination at high concentrations,

430   400/400 μM at 30 minutes and 600/600 μM at 2 h. During the active invasion,

26

431   *Toxoplasma* is the effector cell and the recognition of an unknown component in the

432   plasma membrane of the host cell, is required; this event is determined by the GPI

433   anchored proteins from *Toxoplasma*, being the SAG1 protein the most abundant in the

434   plasma membrane of tachyzoites [13]. The expression of SAG1was reduced in parasites

435   that were treated with DHEA and S-P, while the parasites that were treated with DHEA

436   / S-P maintain the expression of this protein similar to the control; is well known that

437   SAG1 is not the only protein that act in this first process of attachment, due to the Sag1$^-$

438   mutants are still able to invade [13]. The motility is essential for the invasion and it

439   depends of the glideosome complex; in this respect, several proteins that participate in

440   the formation of the glideosome and in its correct function reduced its expression when

441   parasites were treated with DHEA and DHEA / S-P, such as GAP40, profilin and

442   ROM4. GAP40 protein act as an anchor for the rest of the glideosome complex [36]; the

443   role of profilin is to sequester the G actin in order to enhance the polymerization, and it

444   has been demonstrated to be essential for gliding motility and cell cycle in *Toxoplasma*

445   [37]; and ROM4 is a rhomboid protease necessary for the cleavage of the complex

446   MIC2 / AMA1 that is formed for the establishing of the MJ during the invasion process,

447   the correct cleavage of the complex is required for correct reorientation of the parasite

448   and gliding motility [38]. The reduction of the expression of these proteins could

449   explain the diminution in the parasite ability for invade the Hep2 cells monolayers when

450   are treated with DHEA or DHEA / S-P.

451   The analysis in primary cultures of macrophages revealed that high concentrations of

452   DHEA affect the tachyzoites ability to establish in the cytoplasm of the phagocytic cell.

453   In comparison with DHEA treatment, neither the conventional treatment with S-P, nor

454   the combined treatment DHEA / S-P present significant differences, even when

455   combined treatment reduced the extracellular viability. It has been proposed that

27

456 tachyzoites can transform the phagocytic vacuole into a parasitoforous vacuole by two

457 different processes; the first includes the formation of the moving junction at the same

458 time that the parasite is phagocytated, and the second proposal is that once the parasite

459 has been phagocytated, is able to invade the phagolysosomal vacuole [13, 39]. Both

460 proposals imply the fusion of the tachyzoite plasma membrane with the macrophage

461 plasma membrane or with the phagolysosomal membrane; this mechanism involves the

462 secretion of proteins from secretory organelles, such as MIC2 and RON4 [18]. In an

463 interesting way, the protein MIC2 exhibited a higher expression when extracellular

464 tachyzoites were exposure to DHEA, its expression was lower with the exposure to S-P

465 and undetectable in both, the combined treatment and control; and the expression of

466 RON4 was higher in the both treatments that include DHEA compared to the S-P

467 treatment and the control (Table 1). It has been previously reported that extracellular

468 tachyzoites that were exposure to progesterone, inhibit the secretion of MIC2 but didn't

469 affect its expression [34]; this inhibition of the secretion leads on the inhibition of

470 gliding motility. In this work we didn't collected the secretion products, but we must

471 collect in future assays in order to determine if these proteins are secreted or not,

472 nevertheless the increment of MIC2 could due to a failure on its secretion.

473 Complementary assays (not shown) revealed that even if the parasites were established

474 inside of the macrophages, the evasion of the lysis was inhibited; this suggest that

475 DHEA treatment could prevent the block of the phosphorylation of the host Immune-

476 Related GTPases (IRGs) by ROP18 and GRA7 proteins from the parasite, diminishing

477 its ability to escape of lysosomal degradation. Concordantly with this, the expression of

478 GRA7 was reduced when parasites were treated with DHEA, while S-P treatment

479 exhibited a similar expression to the control and in an unexpected way the combined

480 treatment with DHEA / S-P increased the expression of the protein (Table 1). GRA7

28

481   interacts with the ROP18 kinase in a complex that targets the host IRGs mediating

482   macrophage survival and acute virulence; ΔGRA7 strain reduces the virulence in a half

483   and the parasites can't evade the lysosomal degradation [40].

484

485   The effect of the DHEA in the structure of the extracellular tachyzoites resulted in the

486   alteration of the plasmatic membrane structure, the organization of the organelles, the

487   structure of the secretory organelles and cytoskeleton structures. At the higher

488   concentrations (400 and 600 μM) tested for 2 h, tachyzoites looked totally swollen;

489   tachyzoites that were treated with S-P and DHEA / S-P, shown the same structure

490   alterations, at lowest concentration and time tested, except for the swollen shape. The

491   lost of the structure and location of secretory organelles when parasites were treated

492   with DHEA, is consistently with the reduction in the invasion and in the ability to

493   escape of the macrophage lysis; because both mechanisms depend on the secreted

494   proteins from micronemes, rhoptries and dense granules; this effect is also related with

495   the diminution in the expression of these proteins, as was previously discussed.

496   Additionally, GRA3 expression was reduced when parasites were exposed to DHEA

497   and S-P; recently was reported that GRA3 could has a role in the stabilization of the

498   subpellicular cytoskeleton network, as ΔGRA3 strain tachyzoites purified cytoskeletons

499   lost the organization of this structure [41].

500

501   Another three proteins with differential expression regulation worth noting are of

502   diacylglycerol kinase catalytic domain-containing protein, enolase 2 and a cytochrome

503   b5 family heme/steroid binding domain-containing protein. The former, is a protein that

504   is essential for the micronemes correct secretion [42]; this protein increases its

505   expression in all three treatment schemes, as we didn't collect secretion products more

506   experiments should be achieved in order to determine the effect of the hormone in the

507   function of this protein.

508   Enolase 2 besides of being specific of the tachyzoite stage, acts as a transcription factor

509   during intracellular proliferation [43-44]; this protein maintains its expression similar to

510   the control when parasites were expose to DHEA, while its expression was reduced

511   with the S-P and DHEA / S-P treatment. We didn't identify proteins specific of

512   bradyzoite stage, but would be interesting the search of some proteins in order to

513   determine a possible transformation from tachyzoite to bradyzoite stage.

514   The later protein was expressed only in the parasites that were exposed to DHEA. Given

515   its homology to PGRMC1 and 2, proteins known for their roles as progesterone

516   receptors as well as interactions with the family of cytochromes P450 monooxygenase

517   systems (doi.org/10.3389/fphar.2017.00159), it is not surprising to find it associated to a

518   drug metabolism and response function in *T. gondii*. Interaction between this PGRMC

519   homolog and DHEA could potentially block normal activating interactions with CYPs

520   thus preventing the removal of the steroid. Thus, both the experiment in cells, as well as

521   the molecular docking provides evidence for dehydroepiandrosterone to have a different

522   target and effect than S-P.

523

524   Together our results suggest that DHEA can be proposed as a new treatment by itself or

525   in a combined scheme with conventional treatments; however more experiments should

526   be achieved in order to investigate its parasiticide effect *in vivo*.

527

528 ## Conclusion

529 DHEA parasiticide effect could be due to its interaction with the cytochrome b5 family

530 heme/steroid binding domain-containing protein. DHEA treatment reduces the

531 expression of proteins that are essential for the motility and virulence of RH strain

532 tachyzoites and it is likely to block removal of DHEA by CYPs. This leads on an

533 alteration of the ultrastructure of the parasites, the lost of the organelles organization,

534 the lost of structure of the secretory organelles and of the cell shape. These alterations

535 induce a reduction of the invasion ability of the tachyzoites to HEp-2 monolayers; a

536 reduction of the ability to scape of the macrophage's lysis; and a reduction of the

537 viability *in vitro*.

538

Materials and methods

Drugs, reagents and solutions

The DHEA (Sigma chemical Co. Steinheim, Germany) was dissolved in anhydrous

ethanol (Sigma chemical Co. Steinheim, Germany). The sulfadiazine-pyrimethamine

was obtained in its commercial formulation (Bactropin ®, trimethoprim-

sulfamethoxazole 160/800 mg).

Animals

Male Balb-C/Ann mice, between 6-8 weeks of age, used for parasite infection were

maintained in a pathogen-free environment with regulates conditions of temperature,

humidity and filtered air. Animals were fed with autoclaved food and water at libitum;

and maintained according to the Mexican Federal Regulations for Animal Production,

Care and Experimentation (NOM-062-ZOO-1999, Ministry of Agriculture; Mexico

City, Mexico). All efforts to minimize animal suffering and to reduce the number of

animals used were made.

Maintenance and purification of *T. gondii* tachyzoites

Tachyzoites of RH strain was maintained by intraperitoneal (ip) passages in Balb/cAnN

mice. After cervical dislocation parasites were recovered from peritoneal exudates,

washed with 1X PBS (138 mM NaCl, 1.1 mM K2PO4, 0.1 mM Na2HPO4 and 2.7 mM

KCl, pH 7.2) and purified by filtration through 5 μm pore polycarbonate membranes

(Merck Millipore Co. Cork, Ireland).

HEp-2 cell culture

Hep-2 cells (CCL-23), derived from Human larynx epidermoid carcinoma epithelial

cells were obtained from American Type Culture Collection (ATCC). The cellular

culture was maintained in Minimal Essential Medium media (MEM, Gibco, Thermo

563   Fisher, NY, USA), supplemented with 8 % fetal bovine serum (FBS Gibco, Thermo

564   Fisher, NY, USA) and 1 % PES, under 5 % CO_2 atmosphere at 37 °.

Murine macrophages culture

566   Sterile mineral oil (1 mL) was inoculated in the peritoneum of male BalbC/AnN mice,

567   after five days the mice were sacrificed and intraperitoneal macrophages were recovered

568   using 1% glucose-PBS solution; macrophages were centrifuged and pellet was

569   resuspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher,

570   NY, USA), supplemented with 8% of fetal bovine serum (FBS) and 1% of penicillin-

571   streptomycin (PES 10, 000 u/mL, Thermo Fisher, NY, USA). Macrophages were

572   seeded over sterile coverslips in a ratio of 250 X 103 / cm2 and they were maintained at

573   5% CO_2 atmosphere at 37 °.

Macrophage activation

575   Macrophages were washed with fresh DMEM 24 h after they were seeded.

576   Lipopolysaccharides (LPS, Sigma chemical Co. Steinheim, Germany) were added at 30

577   ng/mL final concentration for 1 h, in order to activate the macrophages. Then they were

578   invaded with tachyzoites as described in "Invasion assays" section.

Viability of extracellular tachyzoites

580   Purified parasites (6 X 106 cells) were exposure to increasing final concentrations of

581   DHEA (1, 10 and 100 nM, and 1, 10, 20, 50, 80, 100, 200, 400 and 600 μM), of

582   Sulfadiazine-Pirimetamine (80, 200, 400, 600 and 800 μM) and to combined treatment

583   DHEA/ S-P (80/80, 200/200, 400/400, 600/600, 600/800, 10/600 and 10/800 μM), all

584   drugs were diluted in PBS to final concentrations. The exposition was held for 0.5 or 2h

585   at room temperature (RT) with gentle agitation. The ratio between live and death

586   tachyzoites was measured by exclusion technique with trypan blue (4% Gibco, Thermo

587   Fisher, NY, USA), 300 parasites were counted under an optical microscope

588   (AxioObserve Microscope, Carl Zeiss Mexico). This assay was realized by triplicate in

589   at least three independent assays.

Invasion assays

591   Hep-2 cells were grown on sterile coverslips in MEM medium supplemented with 8%

592   SFB for 24h until the monolayer reach 80% confluence. Tachyzoites were pretreated

593   with the same conditions that for viability assay. Then Hep-2 cells were exposed to

594   tachyzoites pretreated, after 2h of interaction, samples were processed for optical

595   microscopy analysis. Briefly, cells were fixed with 10% formaldehyde 30 min,

596   permeabilized 5 min with 0.1% Triton-X100 and washed with 1X PBS. The samples

597   were staining with haematoxylin – eosin solution (Merck Millipore KGaA, Darmstadt,

598   Germany), washed with 50 % ethanol and slides were mounted with a PBS: Glycerol

599   (1:1) solution. The invasion process was evaluated counting 300 total cells, we

600   considered as invaded cells, every cell that presented at least one parasitophorous

601   vacuole on their cytoplasm. Quantitative analysis was performed in an AxioObserve

602   microscope (Carl Zeiss, Mexico). This assay was realized by triplicate in at least three

603   independent assays.

Induction of changes in tachyzoites morphology by DHEA

605   Extracellular tachyzoites treated with DHEA, S-P or DHEA / S-P at several

606   concentrations, for 30 min or 2 h, were processed for Transmition Electron Microscopy

607   (TEM). Briefly, tachyzoites were resuspended in 2.5 % glutaraldehyde in 1X PBS in

608   gentle agitation for 1 h, washed with 1X PBS, fixed with 1 % OsO4 1 h, and contrasted

609   with 1 % aqueous uranyl acetate 2 h. Samples were dehydrated in increasing

610   concentrations of ethanol (50-100 %), then were embedded in crescents concentrations

611   of LR White resin (London Resin, England, Electron Microscopy Sciences, USA) and

612   polymerized at 4ºC for 36 h under UV lamp. The samples were processed with a

613   ultramicrotome, serial cut were performed of around 10 μm of thickness and mounted in

614   a sample holder, the ultrastructural analysis was performed in a Transmission Electron

615   Microscope JEM200CX 200KV (JEOL Co., Tokyo, Japan), image analysis was

616   performed using the Digital Micrograph program (TM 3.7.0 for GSM 1.2 by the Gatan

617   Software Team).

618   **2D SDS-PAGE**

619   Whole extract of, intact or treated parasites with DHEA (10 μM), S-P (800 μM) and

620   DHEA / S-P (10 / 800 μM) for 30 minutes, was obtained by lysis in 2D sample buffer;

621   extracts were centrifuged at 10 000 rpm, soluble fractions were quantified in a

622   Nanodrop^{TM} 2000 (Thermo Scientific) at 280 nm. 100 μg of whole extracts, contained

623   in 125 μL of rehydratation buffer, were load on ImmobilineTM DryStrip pH 3-10, 7 cm

624   strips (GE Healthcare).

625   After 16 h of passive rehydratation, isoelectric focus was performed in a Protean IEF

626   Cell (Bio-Rad Laboratories, Firmware Version: 1.80) by the supplier specifications.

627   Strips were equilibrated in an equilibrium buffer (6 M urea, 0.375 M Tris-HCl pH 8.8,

628   2% SDS, 20% glycerol) with 0.5% dithiothreitol (DTT) for 10 min, and then with

629   equilibrium buffer with 2.5 % iodoacetamide (IAA) for 10 min; strips were loaded in

630   polyacrylamide precast gels (Mini-PROTEAN®TGXTM Precast Gels 4-20%, Bio-Rad)

631   and electrophoresis was performed at 100 V; then gel were stained with silver nitrate

632   and scanned in a HP Scanjet G4050 scanner.

633   ***In silico* analysis of 2D SDS-PAGE**

634   Images were analyzed in the PDQuest Advanced-8.0.1 program. We determined the

635   spots number for each condition, and then we matched all gels using the control as

636   reference, in order to obtain the matched spots between conditions, their relative

637   expression, as well as, their isoelectric point (IP) and molecular weight (Mr). The spots

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638   that exhibited differential expression between treatments were identified by its

639   molecular weight and isoelectric point using the TagIdent program of ExPASy portal

640   (https://web.expasy.org/tagident/).

641   **Modelling, docking and molecular dynamics of the cytochrome b5 family**

642   **heme/steroid binding domain-containing protein**

643   Initial model generation was accomplished by using the cloned sequence for

644   *Toxoplasma gondii* **PGRMC** and submitting it to Rosetta Homology modeling [45].

645   Resulting models clustered close together for the selection of the best model. However,

646   since the template is a PGRMC1 protein in complex with a heme group (PDB ID

647   4X8Y) [46], these models were refined using UCSF Chimera-Modeller plugin [47-48].

648   Then, their quality was evaluated using Molprobity [49]. The highest quality model was

649   selected to perform ligand docking. Blind docking was performed using Vina 1.1.2 on

650   the LNS supercomputer. All ligands were obtained from the ZINC database and

651   converted to PDBQT format using the GUI provided by Autodock Tools. The receptor

652   was kept rigid during docking. Docking employed a grid of dimensions 40 x 40 x 40

653   with a 1 Å grid size. Exhaustiveness was always set to 1000. Analysis of the docking

654   results was performed in UCSF Chimera. The results presented in Table are the best

655   candidates selected from the consensus score the three best results.

656   **Statistical analysis**

657   Statistical analysis was performed with a variance analysis (ANOVA) of two ways that

658   allowed determined simultaneity the effect of two variables (treatment and exposition

659   time) with the tukey comparation prove. We use the program GraphPad Prism 6,

660   analysis was considered significative different when $p < 0.05$.

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Competing financial interests

The authors declare no competing financial interests.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Saé Muñiz-Hernández: Methodology, conceptualization, project administration and writing.

Carmen T. Gómez-de León: Experimentation, data analysis and writing.

Angélica Luna Nophal: Experimentation.

Lenin Domínguez-Ramírez: Experimentation, data analysis and writing.

689   Olga Araceli Patrón Soberano: Experimentation and data analysis

690   Karen E Nava-Castro: Data analysis.

691   Pedro Ostoa-Saloma: 2D SDS-PAGE methodology and resources

692   Jorge Morales-Montor: Writing (review and editing), data analysis and resources.

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