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Effects of Ginseng extract treatment on preimplantation development *in vitro* and pregnancy in the mouse

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Running Title: American Ginseng Extract *in vitro* and *in vivo* effects on mouse development

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Abstract:

In North America a high proportion of pregnant women use herbal medications, including North American ginseng. This medicinal plant contains a high amount of triterpene saponins (ginsenosides) which are the main bioactive compounds. It is important to assess ginseng's impact on all reproductive functions to ensure the safety of pregnant women and fetuses. In this study, we defined the concentration responsive effects of North American alcoholic and aqueous ginseng extracts on preimplantation development *in vitro* and on pregnancy and post-partum development in the mouse. Two-cell mouse embryos were cultured with 5 different concentrations of whole ginseng root extracts, or ginsenosides Rb1, Rg1 and Re alone, a combinatorial ginsenoside solution and a crude polysaccharide fraction solution. Embryonic development and recovery from treatment was assessed. To investigate *in vivo* effects of ginseng extracts, female mice were gavaged with 50mg/kg/day, 500mg/kg/day or 2000mg/kg/day of either extract (treatment) or water (sham) for 2 weeks prior to mating and throughout gestation. Gestation period, litter size, pup growth and pup sex ratio were evaluated. Oral ginseng consumption did not significantly affect fertility or pregnancy in the mouse. High doses of ginseng (2000mg/kg/day) decreased maternal weight gain. Direct treatment of preimplantation embryos *in vitro* demonstrated that ALC and AQ extract treatment reduced development in a concentration responsive manner, while only ALC extract effects were largely reversible. Treatments with individual or combinatorial ginsenosides or polysaccharide fraction solution alone did not impair preimplantation development, *in vitro*. In conclusion, maternal oral consumption of ginseng has little negative impact on pregnancy in the mouse

however direct exposure to ginseng extract during mouse preimplantation development *in vitro* is detrimental.

Key Words: ginseng, ginsenosides, fertility, blastocyst, pregnancy, assisted reproductive technologies

Introduction:

Ginseng is one of the ten most commonly used herbal medicines in the United States (Bent and Ko, 2004; Chu and Zhang, 2009). Ginseng extracts have reported benefits to the cardiovascular, nervous and the reproductive systems (Attele *et al*., 1999). In the US, 9-45% of pregnant women use herbal supplements during pregnancy (Eisenberg *et al*., 1998; Gibson *et al*., 2001; Tsui *et al*., 2001; Glover *et al*., 2003), with 2.4% ingesting ginseng (Glover *et al*., 2003). Use of herbal products varies from 26-40% of European women during pregnancy (Bishop *et al*., 2011; Nordeng *et al*., 2011). In China, up to 15% of women consume ginseng during pregnancy, as it is believed to be "good for pregnancy and the fetus" (Ong *et al*., 2005). Herbal remedies are often deemed harmless because they are "natural", but this does not in any way ensure that consumption of ginseng is safe for preimplantation development or pregnancy (De Smet *et al*., 2004). Most herbal products carry a warning label to not consume during pregnancy. This is related to the lack of scientific information on safety during pregnancy. Approximately 40 ginsenoside compounds have been identified from the ginseng root (Fuzzati, 2004). Ginsenosides belong to a family of steroids called triterpene saponins (Attele *et al*., 1999) and are either derivatives of the (20S)-protopanaxadiols (PD), (20S)-protopanaxatriols (PT, Tawab *et al*., 2003), acotillol-type or oleanolic acid groups (Fuzzati, 2004). The other major component found in water extract is polysaccharides, which are sugar polymers that have immune-modulatory activity.

To date, research investigating ginseng's impact on reproductive function has demonstrated lower morphological scores for *in vitro* treated rat and mouse post-implantation embryos than for control embryos (Chan *et al*., 2003, 2004; Liu *et al*., 2005,

2006). Conversely, Lee *et al*. (2008) found treatment of early rat embryos with individual ginsenosides resulted in either no significant change to the morphological scores or improvements. In addition, very recent *in vivo* studies have shown that when up to 2000 mg/kg/day (≈200 times the recommended clinical dose) of Korean Red Ginseng extract was administered to female mice, maternal weight, feeding, embryonic implantation and fetal growth were not significantly affected (Shin *et al*., 2010).

It is important to establish whether consumption of ginseng extracts are safe during pregnancy and what effects, if any, direct exposure to these extracts may have on preimplantation development. North American ginseng (NAG) has a much higher concentration of ginsenosides Rb1 and Re, and little Rf compared to Asian ginseng extract (Kitts *et al*., 2000; Sievenpiper *et al*., 2004; Sun *et al*., 2011). Furthermore, the alcoholic (ALC) extract derived from NAG possesses twice the total concentration of ginsenosides as the Aqueous (AQ) extract, specifically 282.5 mg/g versus 138.7 mg/g (Azike *et al*., 2011). In addition, the AQ extract is composed of 15% polysaccharides, whereas the ALC extract has none. Finally, due to so much variability between the compounds found in different ginseng products (Liberti and Der Marderosian, 1978), it is important to study the effects of different ginseng species and extracts on reproduction, which is why the aqueous and alcoholic extracts produced from NAG are the focus of this research.

Materials and Methods

Chemicals

KSOMaa (potassium simplex optimized medium with amino acids) was used for embryo culture medium (EMD Millipore Corporation, Billerica, MA, USA). The Ontario Ginseng

Growers Association provided four-year-old North American ginseng roots, which were extracted to produce the AQ and ALC fractions (Azike *et al*., 2011). The crude polysaccharide preparation was obtained after precipitation from AQ extract in the presence of 40% alcohol (Azike *et al*., 2011). Ginsenosides Rb1, Rg1 and Re were purchased from Sigma (St. Louis, MO. USA).

Animals

All animal care and handling procedures were conducted as approved by the University of Western Ontario, Animal Care and Veterinary Science (ACVS), standard operating procedures that conform to guidelines produced by the Canadian Council for Animal Care (CCAC). Six-eight week old female MF1 mice were purchased from Harlan Laboratories (Indiana, USA) and CD1 mice were purchased from Charles River (Canada). Each female was injected with 7.5 units of Pregnant mares serum gonadotrophin (Folligon, Intervet, Whitby, ON), then 7.5 units of human chorionic gonadotrophin (Chorulon, Intervet) 48 h later and placed with CD1 males for mating. Pregnancy was confirmed the following morning by the presence of a vaginal plug which was considered gestational day one. On gestational day two, the mice were euthanized employing CO_2 asphyxiation, followed by cervical dislocation. Oviducts were dissected and two-cell stage embryos were flushed with M2 flushing medium (Sigma, St. Louis, MO, USA). For all *in vitro* treatment experiments, 20 embryos were cultured in 20 µl drops under oil at 37°C under a 5% O_2, 5% CO_2 and 90% N_2 atmosphere for 48 h.

In vitro Experimental Sequence

Alcohol and Aqueous Ginseng Extracts

Two-cell embryos were washed three times in KSOMaa. Individual stock solutions (10,000 μg/ml) of the alcoholic and aqueous ginseng extracts were made in KSOMaa. These stock solutions were diluted to prepare: 0 (control), 10, 50, 250 and 1000 μg/ml of each extract.

Individual Ginsenoside Rb1, Rg1 and, Re Treatments

Ginsenoside concentrations were calculated to equal the highest actual individual concentrations in either of the AQ and ALC extracts. One mg of ginsenoside Rb1, Rg1 and Re were individually mixed with 1 mL KSOMaa to prepare 1000 μg/ml stock solutions. The stock solutions were then diluted for each individual ginsenoside as follows: Rb1 164.5, 41.1, 8.2, 1.6 and 0 μg/ml; Rg1 13.1, 2.6, 0.66, 0.15 and 0 μg/ml and Re 89.6, 22.4, 4.5, 0.9 and 0 μg/ml.

Ginsenoside Combinatorial Experiments

Two-cell embryos were washed three times in KSOMaa. Ginsenoside concentrations were calculated to equal the actual individual concentrations of Rb1, Rg1 and Re found in the ALC extract. Three individual stock solutions (1000 μg/ml) of purified ginsenoside Rb1, Rg1 and Re were made. The stock solutions were diluted to equal the 257.1 μg/ml concentration found in 1000 μg/ml of the alcoholic extract: which consisted of Rb1 (64%); Rg1 (1%) and Re (35%). This stock was then diluted to provide the five treatment concentrations of 257.1, 64.3, 12.9, 2.6 and 0 μg/ml.

Polysaccharide Fraction

For all polysaccharide fraction (PSF) experiments, CD1 females were used. Fifteen percent of the aqueous extract is composed of PSF, thus this percentage was used to calculate the maximum concentration in 1000 μg/ml AQ extract. A stock solution of 10,000 μg/ml PSF was made and then this stock solution was adjusted to the following concentrations: 150, 37.5, 7.5, 1.5 and 0 μg/ml.

Morphological Assessment

For all *in vitro* experiments, embryo development was assessed after 48 h of treatment. After treatment and culture, the cell number of each embryo was determined and categorized into 2, 4 or 8 cells, 8-cell compacted, morula or blastocyst groups.

Recovery from Treatment

After the morphological assessment following 48 h of treatment, embryos in all experiments were washed three times in fresh ginseng or ginsenoside free KSOMaa and returned to culture for another 72 h in order to determine if recovery from ginseng exposure was possible. After 24 and 72 h, embryos were once again categorized into their specific developmental stages.

***In vivo* experiments**

Animals

In the first two experiments, female MF1 mice (six-eight weeks old) were gavaged for 14 days prior to mating and throughout gestation. For the third experiment, CD1 females were

used, as MF1 mice were no longer commercially available. Three experiments were conducted, females gavaged 50 mg/kg/day (MF1), females gavaged 500 mg/kg/day (MF1), and females gavaged 2000 mg/kg/day (CD1) for 14 days prior to mating and throughout gestation. The experiments consisted of three treatment groups: sham-gavaged water, aqueous extract and alcoholic extract. When administering a dose of 500 or 2000 mg/kg/day, the solutions were prepared 10 or 40 times more concentrated than the 50 mg/kg/day concentration to keep volumes gavaged to a maximum of 0.3 ml total volume/day. Females were gavaged (using standard operating procedures as outlined by Western University's ACVS) once per day for 14 days prior to natural mating and during gestation. The volume gavaged was calculated according to individual mouse weights and adjusted as the mouse gained weight during pregnancy. Females were naturally mated by placing with CD1 males for up to five days. Following detection of a vaginal plug, females were separated from males and placed into individual cages until they gave birth. N values were 4-6 pregnant mice per treatment group per experiment.

Outcome Assessment

Once litters were born, day of birth, litter size, and gestation period were determined for each group in each experiment. Pups were individually weighed every four days until weaning on post-partum day 20. Offspring sex was determined at weaning.

Statistical Analysis

Statistical analyses were done with GraphPad Prism version 4.0c software or SigmaStat 3.5 (Jandel Scientific, San Jose, CA). Each culture experiment was repeated a minimum of

three times using embryos collected from replicate groups of mice. Data are presented as the mean ± standard deviation (SD). For *in vivo* experiments, litter size, gestation period and proportion of male pups, statistical analyses were conducted with ANOVA. Where the conditions of normality or equal variance were not met, the Kruskal-Wallis non-parametric test on ranks was used. To correct for litter size, the mean pup or male/female pup weight was used for each litter in the analysis. We report on the results of one-way ANOVA for weights on the day of birth and at weaning. A two-way ANOVA analysis was used to examine the effects of treatment and sex on pup weight at weaning (D20). For maternal weights in the 2000 mg/kg/day experiment, during group housing in the feeding period before pregnancy, one-way ANOVA was used to detect treatment differences. During lactation, two-way ANOVA with repeated measures was used to evaluate maternal weights, multiple comparisons performed when effects were significant, n values were 5-6. Data were subjected to a reciprocal transformation $p < 0.05$ was considered to be statistically significant.

Results

Effect of Aqueous Ginseng Extract Treatment on Mouse Preimplantation Development *in vitro*.

Aqueous ginseng extract treatment resulted in a concentration-dependent inhibition of preimplantation development (Figure 1A). As the concentration of AQ extract increased, the number of embryos developing to the morula and blastocyst stages was significantly reduced ($p<0.05$). Development was increasingly blocked at the two-cell and four-cell stages (Figure 1A). At 1000 μg/ml, only 1.5% of embryos developed to the morula stage,

whereas 71.7% of control embryos progressed to the morula stage after 48 hours of culture. Embryos treated with 10 μg/ml of the aqueous extract displayed the greatest recovery from treatment as blastocyst formation increased to nearly 80% blastocysts after 72 h of recovery time (Figure 1B). Exposure to all higher aqueous concentrations resulted in no development to the blastocyst stage at 48h of culture ($p<0.05$) and very limited recovery compared with the control (Figure 1B).

Effect of Alcoholic Ginseng Extract Treatment on Mouse Preimplantation Development *in vitro*.

Treatment with the alcoholic extract resulted in similar outcomes as that of the aqueous fraction, with concentrations of 50 and 250 μg/ml significantly ($p<0.05$) increasing the numbers of embryos blocked in development at the two-cell stage (Figure 2A). Paradoxically, treatment with 1000 μg/ml did not result in developmental arrest (Figure 2A). All doses however resulted in decreased blastocyst development at 48h of culture ($p<0.05$). Embryos treated with 10, 50 and 1000 μg/ml of ALC extract recovered from treatment by displaying no significant difference in blastocyst formation after 72 hour recovery period (Figure 2B). Interestingly, the developmental blockade resulting from treatment with 250 μg/ml ALC extract was only marginally reversed with 29.4% of treated embryos developing to the blastocyst stage, as opposed to 97.1% for control after 72 h of recovery time (Figure 2B).

Effect of Individual and Combinatorial Ginsenosides Rb1, Rg1, Re and Polysaccharide (PSF) Fraction Treatment on Mouse Preimplantation Development *in vitro*.

Experimentation using the highest Rb1 concentration found in our extracts resulted in a significant decrease ($p<0.05$) in development to the morula stage between the control and 8.2 μg/ml of Rb1 (Figure 3A). However, following washing and recovery period, there were no differences in blastocyst development at 24h and 72h (Figure 3B). Embryos exposed to even the highest Rg1 concentrations found in our extracts progressed normally to the blastocyst stage (Figures 4A, B). In experiments conducted with ginsenoside concentrations of Re found in our preparations, no significant variations in embryo development between the control and treatment groups were observed (data not shown). Likewise experiments using combinatorial ginsenoside concentrations resulted in no significant impairment in development to the blastocyst stage (data not shown). Finally we treated two-cell stage embryos with polysaccharide fraction concentrations up to that found in the AQ fraction and also did not observe any significant impairment of development to the blastocyst stage (data not shown).

Effects of oral consumption of either Ginseng Extract on Pregnancy and Post-Partum Development in the Mouse

Females Gavaged with 50 and 500 mg/kg/day Ginseng Extracts

Parturition was checked twice daily (day of copulation plug = D1), the gestation period and litter size were determined (Table 1). No significant differences were observed

between treatment groups for litter size or sex ratio in any of the gavage treatments (Table 1). Pup weights were determined by weighing 247 individual pups (50 mg/kg/day) and 212 pups (500 mg/kg/day). There were no sex-specific pup weight differences at weaning due to treatment (data not shown).

Females Gavaged with 2000 mg/kg Ginseng Extracts

Weight of the females were not significantly different at the start of the treatment (Figure 5A), but after one week the ginseng groups weighed less than the SHAM group ($p<0.05$, Figure 5A). Ginseng treated females maintained this difference throughout the feeding period. For individual pregnancy (Figure 5A) days, there were significant effects due to treatment, all $p < 0.05$. By the last gestational day SHAM animals weighed 7-8g more than the ginseng treated females (Figure 5B). For repeated lactation weights (Figure 5B), there were significant differences due to treatment ($p<0.01$) and days ($p<0.01$). During lactation, The AQ extract group weighed less than the SHAM animals (Figure 5B). The average number of pups per litter on the day of birth did not vary significantly between treatment groups (Table 1). To evaluate pup growth and development, 173 pups were individually weighed every four days to Day 20. Sex ratio of the pups did not differ among groups (Table 1).

Discussion

There is a need to investigate the safety of North American ginseng consumption on preimplantation development, pregnancy and post-partum development as assessed in this study. We have discovered that AQ and ALC North American ginseng extract

treatments caused a concentration dependent inhibition of development when applied directly to preimplantation embryos in culture, with the AQ extract causing more permanent effects. Exposure to individual ginsenosides, Rb1 and Rg1, did not produce lasting effects *in vitro*. Importantly, however, pregnancy was unaffected with orally administered NAG extracts up to 2000 mg/kg/day. This study indicates that direct exposure to ginseng extracts is detrimental to the completion of preimplantation development.

The effect of ginseng and ginsenosides on postimplantation embryos has been examined (Chan *et al*., 2003, 2004; Liu *et al*., 2005, 2006; Lee *et al*., 2008), however, few studies have investigated the direct influence on preimplantation embryo development.

Our *in vitro* study shows differences between treatments with the two types of extracts. Excluding embryos treated with the 250 μg/ml dose, all other groups treated with the ALC extract recovered from treatment and progressed onto the blastocyst stage after recovery, in contrast to the irreversibly decreased development in embryos treated with higher doses (> 50 μg/ml) of the AQ extract. This difference is likely not simply due to total ginsenoside concentrations, as on a per weight basis ALC > AQ, but due to a varying compositions of ginsenoside or non-ginsenoside compounds.

In conclusion, the detrimental effect observed on embryonic development induced by treatment with the AQ and ALC ginseng extracts is concentration dependent, and direct embryo exposure to ginseng extracts is embryotoxic. Furthermore, once removed from treatment, embryo recovery is limited for AQ extract treatments greater than 10 μg/ml as most embryos in AQ treatments did not resume development if arrested at the two-cell and four-cell stages. In contrast, embryos treated with ALC extracts displayed a greater recovery from treatment than did the AQ treated embryos. We observed that the ALC

extract displayed a concentration specific increase in the blockade of development at the 2-cell stage up to 250 μg/ml. This concentration dependent effect however did not occur at the highest dose used (1000 μg/ml). This outcome is puzzling, but the effect is reminiscent of high ligand concentration effects on receptor desensitization and thus ligand/receptor signaling. Our study in no way defines the active component in the extract that is facilitating this outcome. We simply speculate that the higher dose induced reduction in 2-cell developmental blockade allowed greater numbers of embryos to progress to the blastocyst stage and fully recover from treatment. Clearly there are some important differences in outcomes observed between direct treatment of early mouse embryos with AQ and ALC extracts *in vitro*.

In our study, we used the maximal Rg1 and Rb1 concentrations found in our extracts, but blastocyst rates were similar for all doses compared to the control. Liu *et al*., 2006 found that Rg1 remarkably decreased the total morphological score of post-implantation mouse and rat embryos when concentrations of 30 and 50 μg/ml were tested. Future studies investigating the effects of Rg1 on early development should consider the use of higher concentration treatments. Re is the second most prominent ginsenoside found in NAG extracts and is representative of the PT group. Re exerts a strong postimplantation embryotoxic effect starting with concentrations of 50 μg/ml (Chan *et al*., 2004). By contrast, our experiments displayed no significant delays in preimplantation embryonic development *in vitro* between the control and concentrations up to 89.6 μg/ml Re. Thus the overall conclusion from the single agent and combinatorial treatment experiments or PSF tested is that none negatively affected mouse preimplantation development. Thus the impact on preimplantation development observed from AQ and ALC treatment must either

result from an individual component not yet identified, or the collective effects of several of the ginseng extract components adversely affecting early embryo development. Our results are the first to indicate that direct exposure to ginseng extracts is detrimental to preimplantation development.

These negative *in vitro* outcomes with whole extracts contrast the findings from *in vivo* studies investigating the influence of Red Korean Ginseng treatment two weeks before mating and throughout gestation on pregnancy in the mouse (Shin *et al*., 2010). Due to these discrepancies in outcomes it was essential to conduct *in vivo* experiments using NAG on pregnancy and post-partum development in the mouse. In this study doses of 50 mg/kg/day to an extremely high dose of 2000 mg/kg/day NAG (≈ 200 times the recommended daily dose of 1-2g) were investigated. Even at this dose, we observed no significant differences in the gestation period, litter size and sex ratio of mice weaned on post-partum day 20. Females lost weight during the feeding period and gained significantly less weight during pregnancy, remaining lower through much of lactation, especially in the aqueous group, which may suggest a toxicity or satiety issue. Ginseng preparations may decrease post-meal blood glucose (Sievenpiper *et al*., 2004; Dascalu *et al*., 2007) or decrease gut uptake of sugars (Onomura *et al*., 1999). Most recommended levels for ginseng product consumption range from 1-2 g per day, indicating that a 50 kg woman would have to ingest 100 g of ginseng daily to equal the 2000 mg/kg/day level that we investigated. The 50 mg/kg/day dose is much closer to realistic consumption levels.

We are intrigued by the fact that our *in vivo* results contrasted dramatically with the direct effects of NAG on preimplantation embryos. We believe this may be due to the fact that the concentrations of ginseng directly affecting embryos *in vivo* are unknown due to

uncertainties regarding ginseng compound absorption, metabolism and renal clearance rate after consumption in the human. In light of the high water solubility of ginsenosides, and their low oral bioavailability, it is very likely that oral consumption results in very little embryonic exposure to ginseng compounds due to the low trans-placental transfer.

Overall our results support a cautionary approach to determining whether ginseng extract consumption has a negative influence on pregnancy and post-partum development.

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Figure Descriptions:

Figure 1: Development of 2-cell mouse embryos after 48 h treatment with aqueous extract. 2-cell embryos were flushed from the oviduct and placed into culture with 0-1000 µg/ml aqueous extract for 48h **(A)** and up to 72h recovery **(B)**. Data are presented as the mean ± SD, representative of three independent replicates. Significant differences are represented by different superscripts ($p \leq 0.05$).

Figure 2: Development of 2-cell mouse embryos after 48 h treatment with alcoholic extract. 2-cell embryos were flushed from the oviduct and placed into culture with 0-1000 µg/ml alcoholic extract for 48h **(A)** and up to 72h recovery **(B)**. Data are presented as the mean ± SD, representative of three independent replicates. Significant differences are represented by different superscripts ($p \leq 0.05$).

Figure 3: Development and recovery of 2-cell mouse embryos treated with ginsenoside Rb1. 2-cell embryos were flushed from the oviduct and placed into culture with 0-164.5 µg/ml Rb1 for 48 h (**A**) and up to 72h recovery (**B**). Data are presented as the mean ± SD, representative of three independent replicates. Significant differences are represented by different superscripts ($p \leq 0.05$).

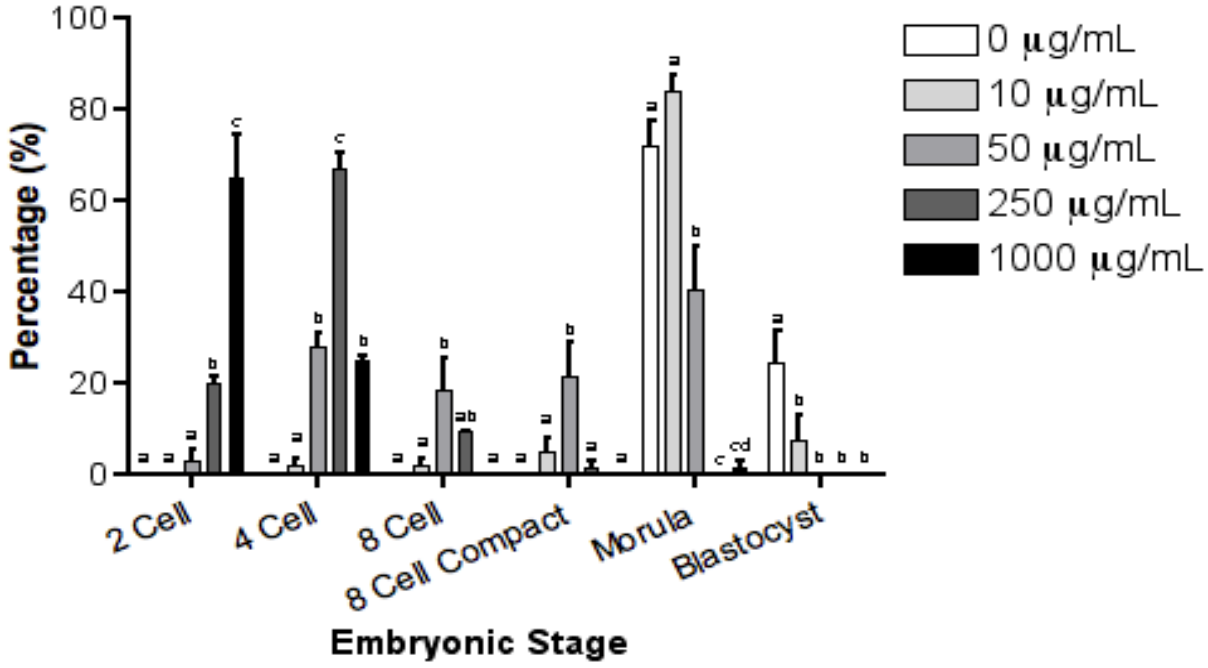
Figure 4: Development and recovery of mouse embryos following treatment with ginsenoside Rg1. 2-cell embryos were flushed from the oviduct and placed into culture with 0-13.1 µg/ml Rb1 for 48 h (**A**) and up to 72h recovery (**B**). Data are presented as the

mean ± SD, representative of three independent replicates. Significant differences are represented by different superscripts ($p \leq 0.05$).

Figure 5: Effect of ginseng treatment (2000 mg/kg/day) on weight during the pre-breeding, pregnancy and post-partum periods. Female mice were gavaged with water (SHAM) or 2000 mg/kg/day of either AQ or ALC ginseng extract for 2 weeks prior to mating and throughout gestation. Weight gain is reported during pre-breeding (**A**), gestation (**B**) and lactation periods **(B)**. Differences in weight between treatment groups are presented as the mean ± SD, representative of 5-6 independent replicates. Significant differences between the SHAM control and ginseng treatments are represented by different superscripts ($p \leq 0.05$).

Figure 1

A **48 h treatment with aqueous extract**



B **72 h recovery after treatment with aqueous extract**

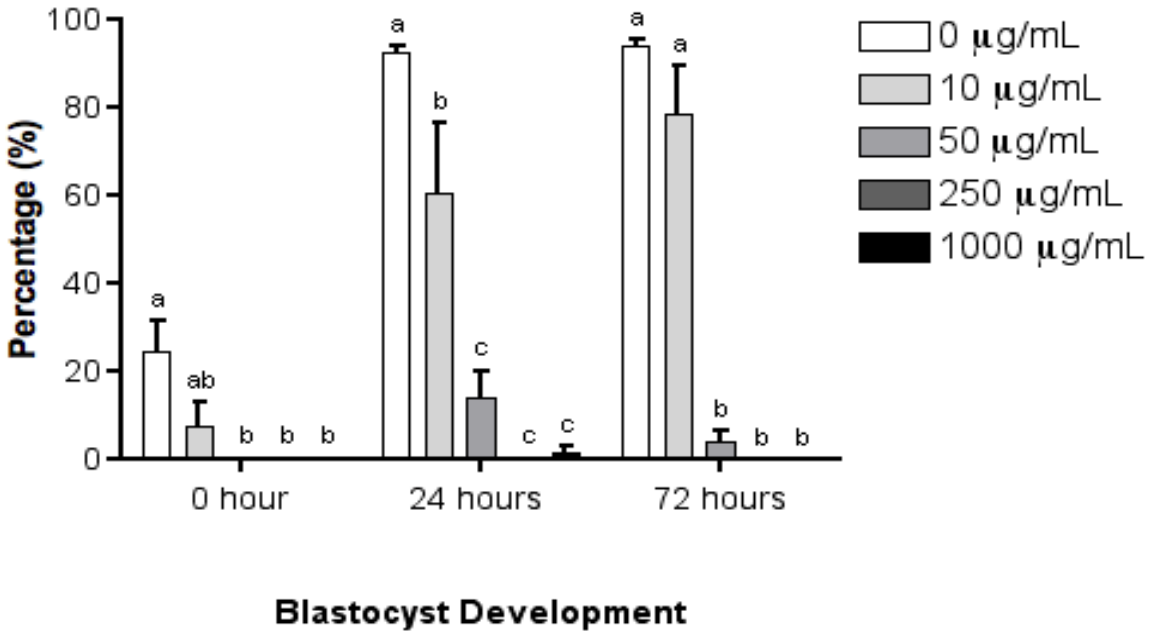
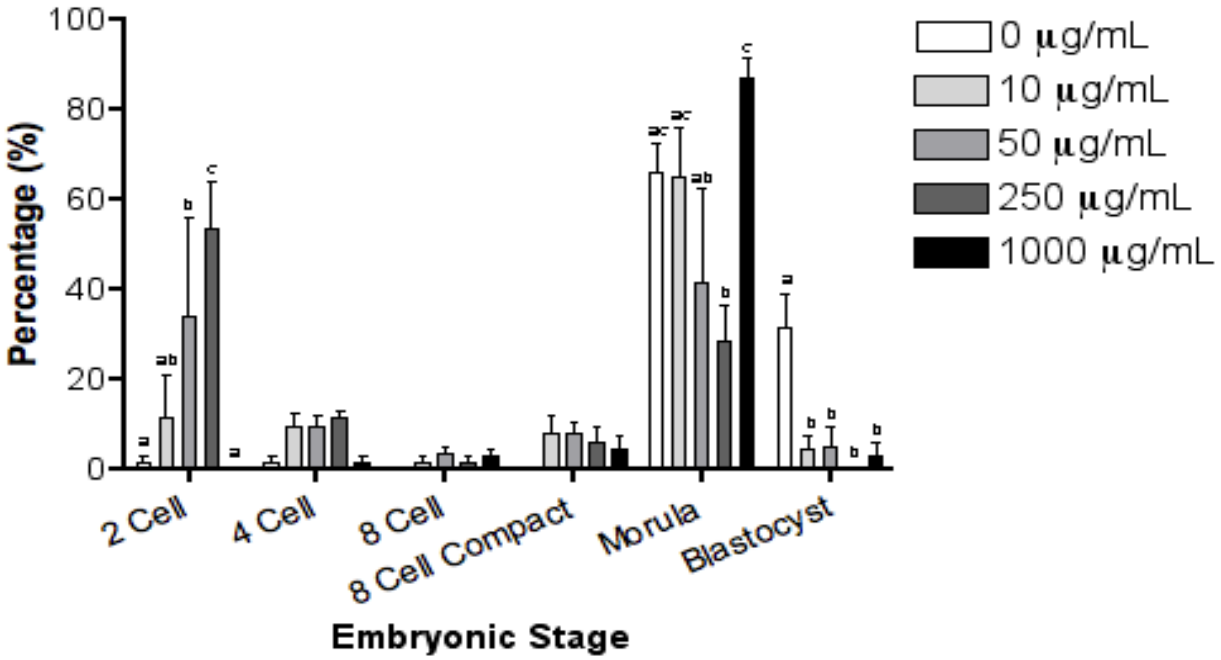


Figure 2

A **48 h treatment with alcoholic extract**



B **72 h recovery after treatment with alcoholic extract**

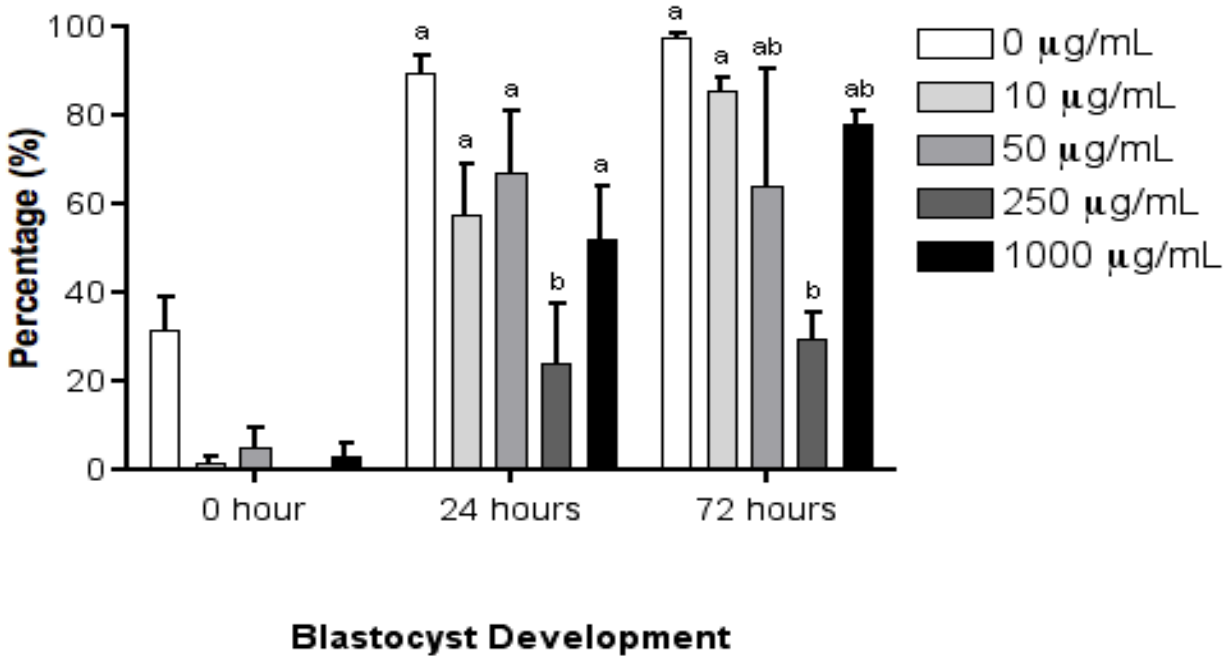
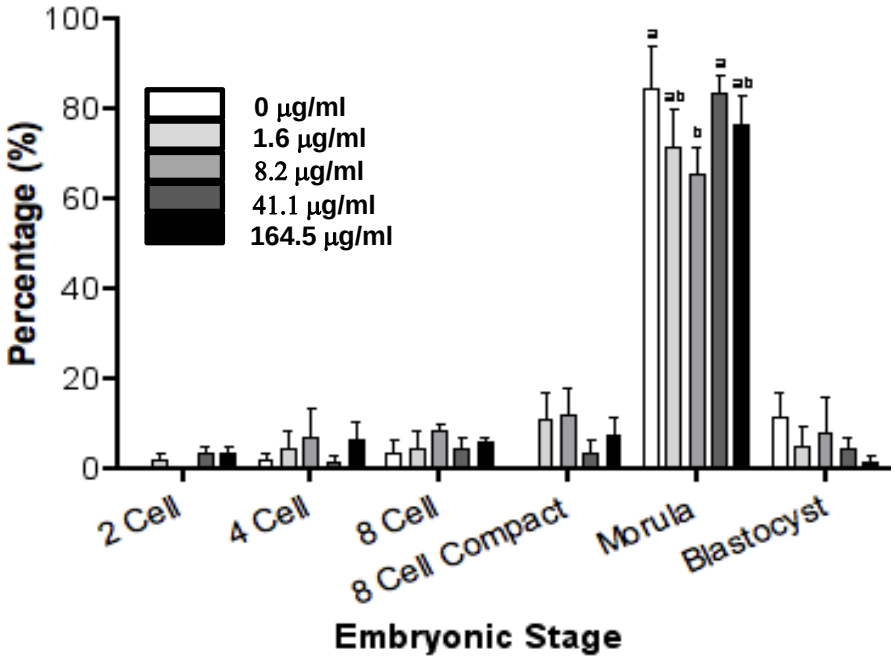


Figure 3

A 48 h treatment with Rb1



B 72 h recovery after treatment with Rb1

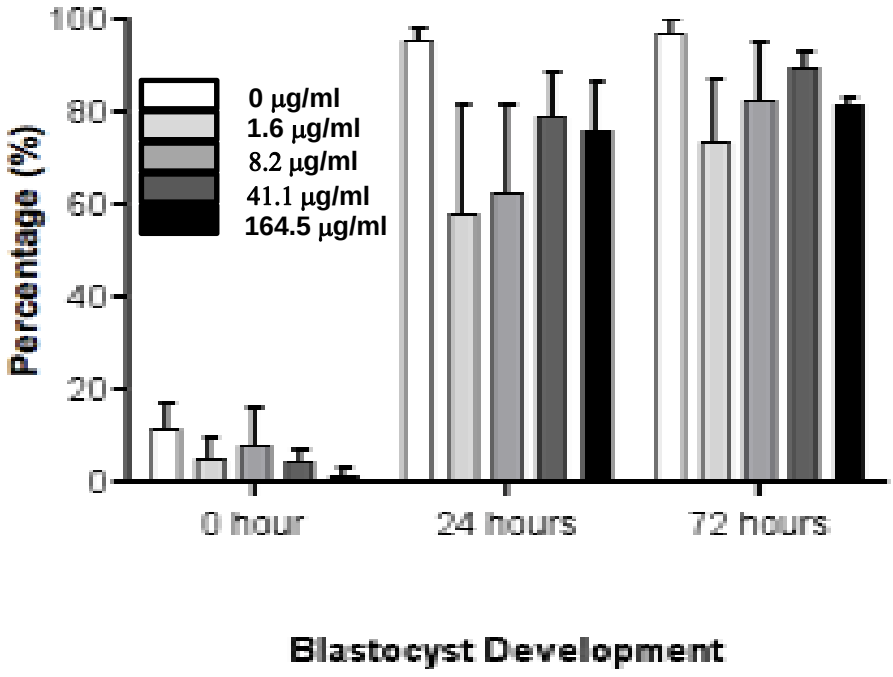
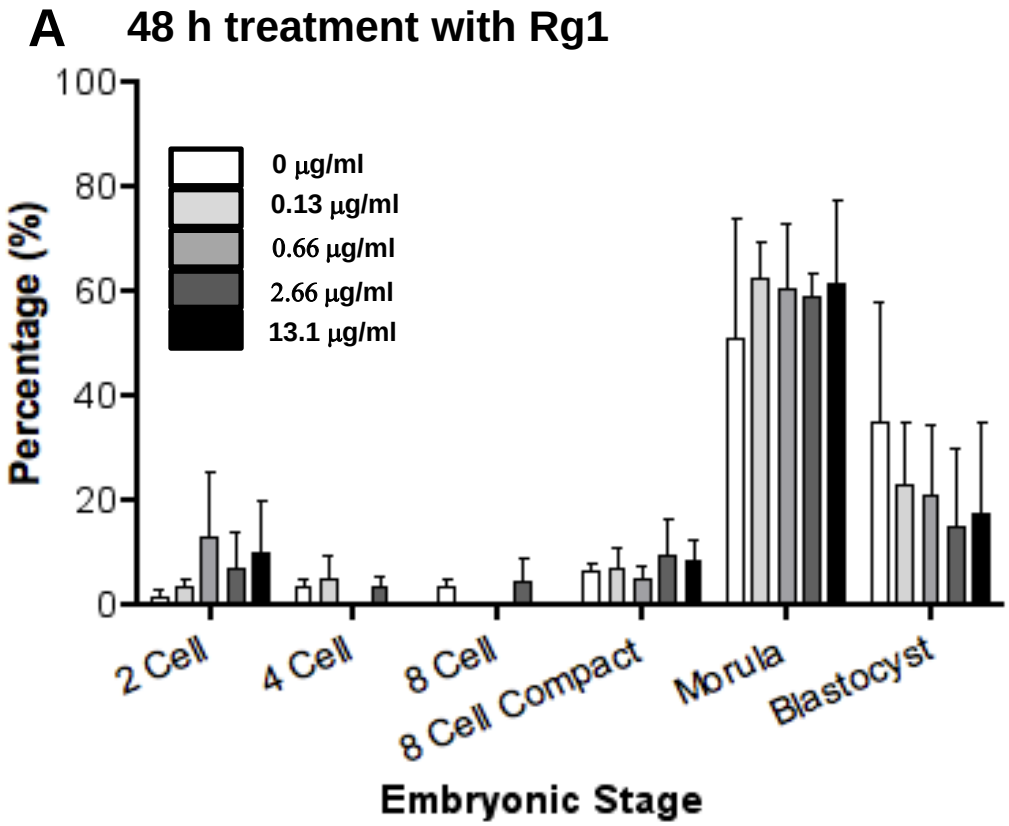


Figure 4


A
48 h treatment with Rg1
100
80
60
40
20
0
Percentage (%)
0 μg/ml
0.13 μg/ml
0.66 μg/ml
2.66 μg/ml
13.1 μg/ml
2 Cell
4 Cell
8 Cell
8 Cell Compact
Morula
Blastocyst
Embryonic Stage

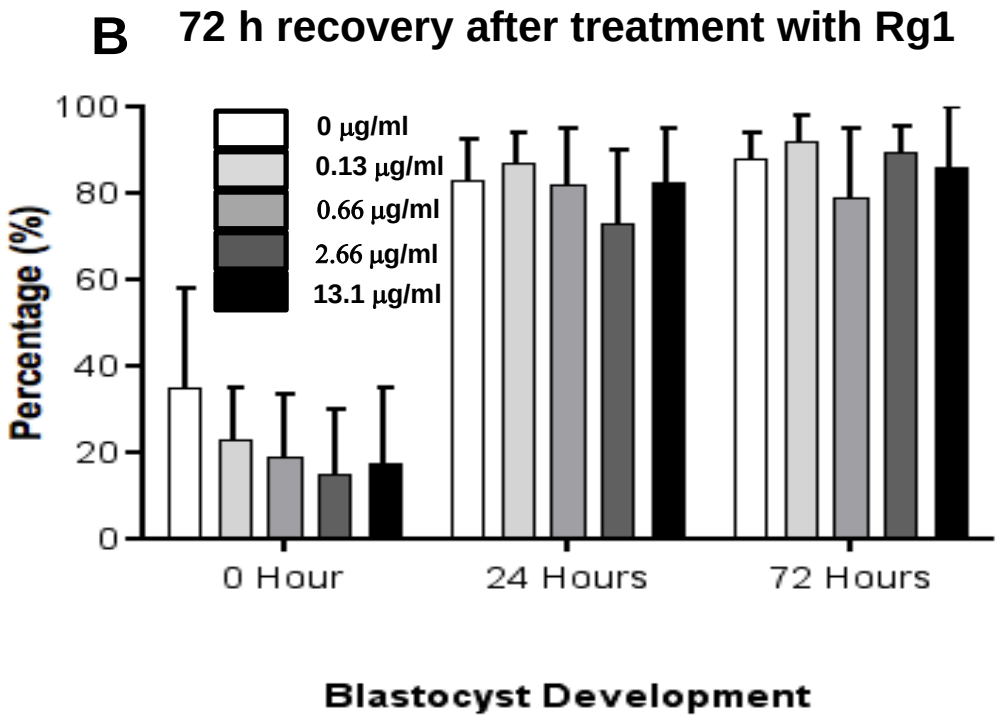
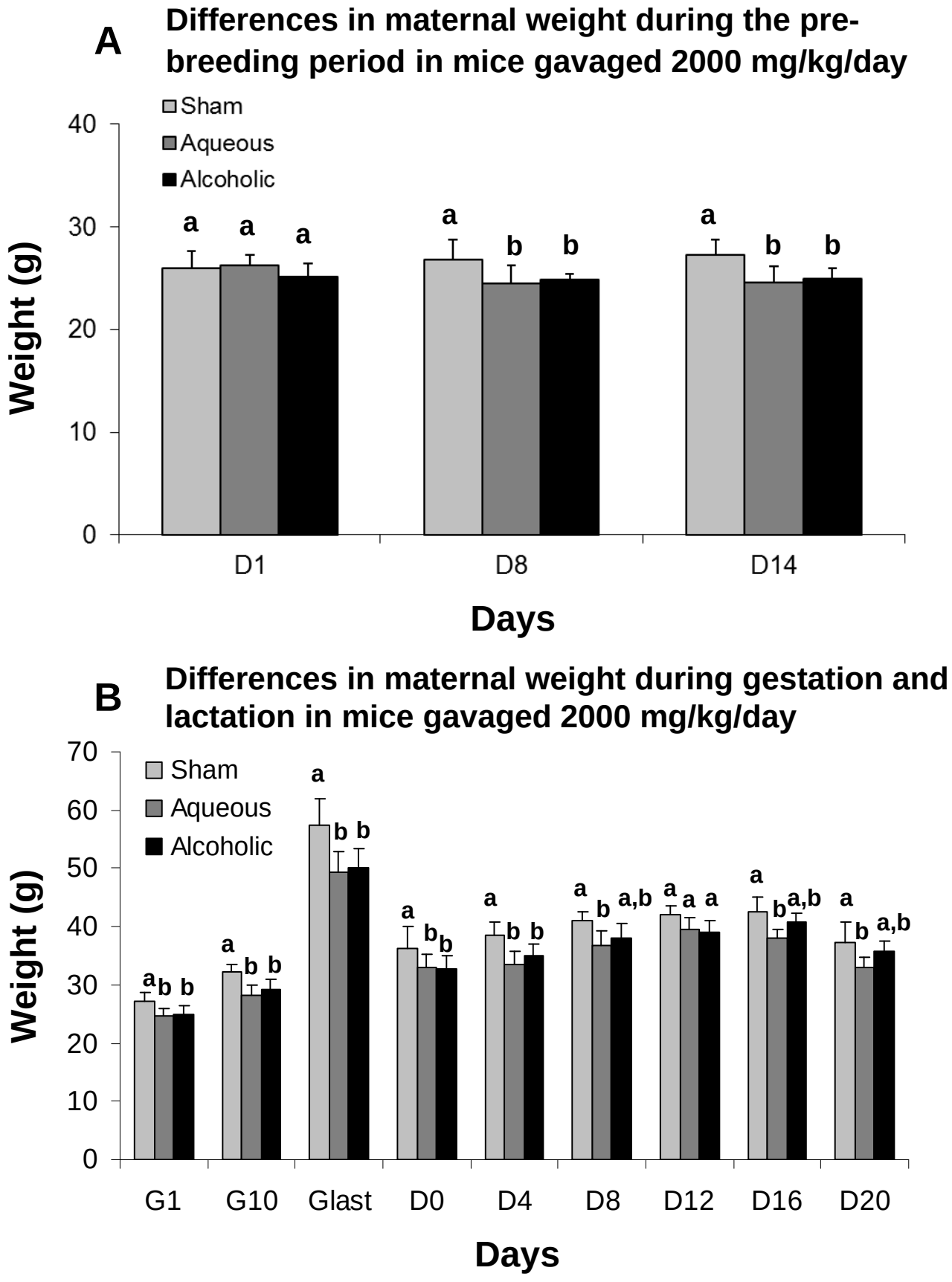

B
72 h recovery after treatment with Rg1
100
80
60
40
20
0
Percentage (%)
0 μg/ml
0.13 μg/ml
0.66 μg/ml
2.66 μg/ml
13.1 μg/ml
0 Hour
24 Hours
72 Hours
Blastocyst Development

Figure 5


A
Differences in maternal weight during the pre-breeding period in mice gavaged 2000 mg/kg/day
Sham
Aqueous
Alcoholic
Weight (g)
0
10
20
30
40
a a a
a b b
a b b
D1
D8
D14
Days
B
Differences in maternal weight during gestation and lactation in mice gavaged 2000 mg/kg/day
Sham
Aqueous
Alcoholic
Weight (g)
0
10
20
30
40
50
60
70
a b b
a b b
a b b
a b b
a b b
a b,b
a a a
a b,b
a b b
G1
G10
Glast
D0
D4
D8
D12
D16
D20
Days