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RESEARCH **P**APER

Evaluation of homeopathic drugs on Glucocorticoid induced osteoporosis (GIOP) zebrafish model

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Background: Homeopathic remedies are proposed as source for traditional preventive medicines. In this context our study focuses to evaluate efficacy of homoeopathic drug with reference to GIOP in Zebrafish vertebrate model. The overall approach behind this study was to determine whether homeopathic drugs can be used along with of allopathic medicines as an alternate therapy. **Methods**: In the present study, an attempt is made to find out the efficacy of homoeopathic medicines- *Argentum Metallicum, Calcaria Carbonica* and *Sepia* in two dilutions 6CH and 30 CH on GIOP model of zebra fish in two dilutions. Exposure was studied at 3 different points - 4, 14 and 28 days exposure (treatment with test drugs) and staining intensities were measured using Image J software. Control groups used were untreated control larvae, GIOP model untreated post 11 dpf and GIOP model treated with Alendronate. The medium was changed every day at the same time. Staining intensities measured for Alizarin Red and Calcein dye stained images. **Results**: Staining intensities of Alizarin red and Calcein staining of the treated group on statistical analysis showed that the means of GIOP and other treated groups were not statistically significant at $p<0.05$ except in the case of 28 day study for *Calcarea Carbonica* 30C and *Sepia* 30C. Though screening was processed using Alizarin red and Calcein staining, our quantification screening with calcein labeling indeed facilitated the process and diminished labor of handling. **Conclusion:**Our findings show that, despite some physiological differences between mammals and teleosts, the zebra fish represents an effective model for screening of bone defects. This model has been used for preliminary studies on homeopathy drugs prescribed for treatment of Osteoporosis. The proposed calcein staining protocol can represents a powerful tool for *in vivo* monitoring of mineralized structures.

Key words : Homeopathy, Osteoporosis, Zebra fish, Prednisolone, Alizarin red, Calcein

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INTRODUCTION

As India is emerging as one of the leaders in Complementary Alternate Medicine (CAM) and is promoting a pluralistic health care delivery system. Homeopathy is an important component of this mission as also highlighted by AYUSH.Basic research in Homoeopathy is in its exploratory stage with generating scientific plausibility of ultra-dilutions and further understanding the mechanisms of their action (WHO, 2016, Central Council for Research in Homoeopathy, 2013). The number of preclinical studies (*in vitro* and*in vivo*) has been conducted in the past aimed at evaluating the pharmacological activity and/or efficacy of some homoeopathic remedies under potentially reproducible conditions (Bellavite *et al.*, 2006). However, the

translation of knowledge from preclinical studies to the clinical level has a gap, due to the limitations of *in vivo* disease models (Vandamme, 2014). The*in vitro* studies and clinical researches need to be backed by more research done on the *in vivo*/biological/animal models. Such research must include laboratory models that mimic the clinical application of homoeopathic substances and facilitate research regarding the central issues of Homoeopathy (Van Wijk and Albrecht, 2007). The drug development process is considered costly and inefficient (Gorman and Bredan, 2007). A crucial gap exists between *in vitro* and *in vivo* when validating mechanism of drugs. The low throughput of mammalian models creates a major bottleneck to evaluate the numerous "hits" identified from cell-based screening. Zebrafish could enhance preclinical drug screening by its strategic placement between cell-based and mammalian models along the drug development pipeline (George *et al.*, 2011 and Fleming and Alderton, 2013). The present study was designed to explore the possibility to test the effects of homeopathic remedies on Glucocorticoid induced osteoporosis (GIOP) zebra fish model (Bareett *et al.*, 2006). Zebra fish (*Daniorerio*) are a great model for bone studies as they have biological structures and properties similar to humans, which suggests that they may be used as a model to study mineralization characteristics of the human Haversian system, as well as human bone diseases (Pasqualetti *et al.*, 2015; Apschner *et al.*, 2011 and Westerfield, 2000). They are recognized models to study skeletal development and regeneration (Westerfield, 2000 and Ge *et al.*, 2006). The development of the skeleton can be observed at very early stages since embryonic/larval zebra fish remain translucent during the first important steps of skeletal development (Kimmel *et al.*, 1998).

The present invention relates to a novel method for visualizing normal and defective bone development with alizarin red andlive staining by calcein dye on exposure with prednisolone 10ug/ml in the media. In addition, zebra fish osteoporosis model was successfully used to verify the effect of 3 homeopathy drugs medicines in two dilutions used to study the effect on osteoporosis model.

RESEARCH METHODOLOGY

Embryo collection:

All experiments conducted in accordance of the Institutional animal ethics clearance from MGM. Experiment conducted during the year 2016-17. Adult zebra fish from an existing stock at the Central Research laboratory, MGM Medical College, MGMIHS, Navi Mumbai were maintained in 20 L glass aquaria (28 ± 1 °C; 12:12 light-dark) which contained charcoal filtered tap water. Fish were fed twice daily. Males and females were separated prior to spawning when they were mixed within a breeding net allowing collection of embryos (Nicenboim *et al.*, 2015)

Exposure protocol:

As per OECD Guidelines Zebrafish Embryo Toxicity (FET) assaycarried out to calculate the effective dose of Prednisolone(Sigma M0639) that can be administered to develop the osteoporotic model. Water and embryo medium were also tested and used as a control. All embryos were derived from the same spawns of eggs for statistical comparison between control and treated groups. Healthy embryos were grown in optimum temperatures in normal condition. For the purpose of experiment 5dfp larvae were used and exposed in 24-well culture plates (04 embryos in 4 ml solution/well). Each group had four replicate wells. Each experiment was replicated three times. At all stages, the developing embryos and larvae were maintained at 28°C in embryo medium.

For valid experiments, fertilized eggs were obtained only from spawns with a fertilization rate higher than 90%. In all experiments, dead embryos and larvae were removed from the 24-well plates every 12 h. For development of model for osteoporosis based on our toxicity screening, we found the effective concentration (EC) of 10 µg/ml prednisolone can be used for inducing osteoporosis in zebrafish larvae. Qualitative study of cartilage and bone malformations was assessed in a semi-quantitative approach form - normally developed, minor malformations, strong malformations, severe malformations and or no longer detectable and skeletal elements. Alizarin red staining, Calcein labeling quantitatively confirmed using Image J software shows bone loss in prednisolone treated larvae using 10ug/ml as the optimum dose. We confirm that the Institutional Animal ethics of MGM Medical College, MGMIHS and Navi Mumbai have approved our study (Gupta *et al.*, 2016).

Homeopathy drugs:

Medicines (potentized hydro alcoholic solutions) with

different potencies (6CH and 30CH) based *Calcarea carbonica*, *Argenticummetallicum* and*Sepia* were obtained from Schwabe India (authorized producing house in Mumbai certified by Good Manufacturing Practices (GMP) and International Organization for Standardization (ISO). The remedies were stored in brown-colored glass containers at room temperature away from sunlight. Alendronate (*PHR1599 SIGMA-ALDRICH)* stock solution (200µl/ml) in DMSO was stored in aliquots at -20°C for maximum one month.

Homeopathy exposure on the GIOP model of Zebra fish:

GIOP model was exposed to the 3 test medicines mentioned above. Exposure was studied at 3 different points - 4, 14 and 28 days exposure and staining intensities were measured using Image J software. 3 control groups were used:

– Untreated control larvae
– GIOP model untreated post 11 dpf and
– GIOP model treated with Alendronate.

The medium was changed every day at the same time.

Bone matrix vital staining :

Evaluation of the bone matrix profile of the prednisolone treated larvae was performed using Alizarin red (Sigma) (Vilmann, 1968 and Springer and Johnson, 2000) and Calcein (Sigma) (Du *et al.*, 2011 and Recidoro *et al.*, 2014) live staining respectively, in accordance with previously published papers (Kimmel *et al*, 1998). 5 dpf larvae were treated with prednisolone for 6 days and later rescue experiment performed using the homeopathy medicines at 3 different time points as mentioned above. At 11 d.p.f., 15 dpf, 25 dpf and 39 dpflarvae were labeled in vivo for demonstration of skeletal mineralization using Calcein live staining and remaining larvae fixed and processed for whole mount skeletal staining using Alizarin red staining.Images were obtained on EVOS FL AUTO imaging microscope using the Green filter for Calcein staining and normal bright field for Alizarin red stained larvae.

Statistical analysis :

All experiments were repeated three times independently. Data were recorded as the mean with the standard deviation (SD). For the embryo/larval bioassays, two tailed paired student t test was used with group having equal variance, at 0.05 significance level to detect significant differences between the control and treated groups.

RESEARCH FINDINGS AND ANALYSIS

We have studied the effects of differing concentrations of a Prednisolone on zebra fish development. Embryos were exposed continuously from 5dpf to 10 dpf. They were then assessed for bone deformities at 11 dpf. The exposure to Prednisolone induced a delay in hatching compared with the controls in a concentration-dependent manner. After exposure

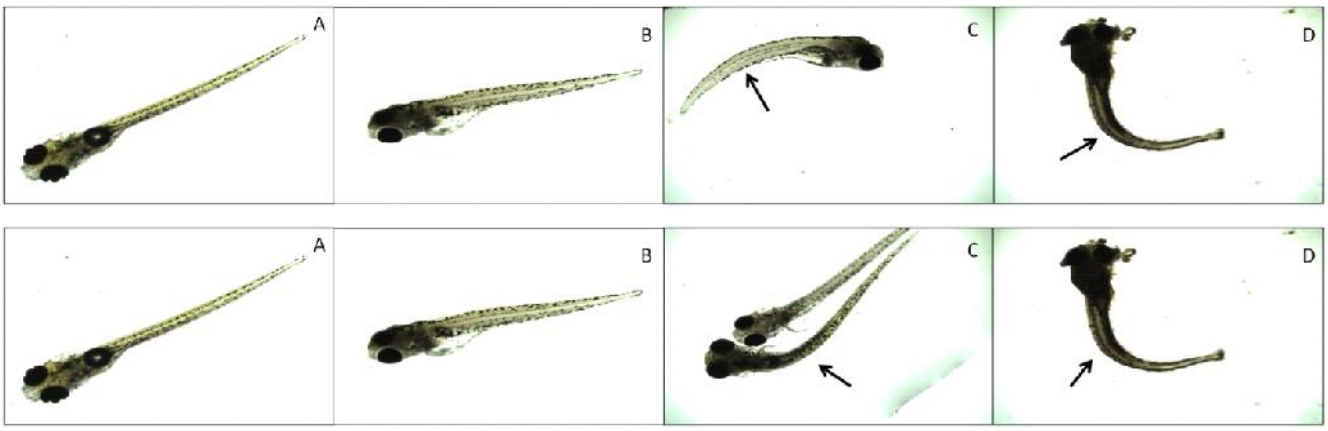

Fig. 1 : Zebra fish larvae observed under the stereomicroscope (A) control larvae 11 dpf (B) Larvae treated with 5ug/ml of prednisolone; no visible deformity evident. (C) Larvae treated with 10ug/ml of prednisolone; curvatures of spine are pronounced , arrows indicate curvature of the spine (D) Larvae treated with 20ug/ml of prednisolone; curvatures of spine are highly pronounced affecting viability, arrows indicate extent of curvature of the spine

with increasing concentrations of Prednisolone skeletal malformations can be observed without histological assay because of the transparency of embryos of*D. rerio* (Fig. 1).

We found the development of the calcified skeletal structure in control group appear in a progressive fashion from head to tail. Calcified structures in the head (*i.e.* the jaw) developed first, which were then followed by the axial skeleton in the trunk. Interesting to note was that there appeared to be two domains in the calcification of vertebrae within the axial skeleton. The first three vertebrae were in the first domain; the rest being in the second domain (Bird and Mabee, 2003, Kimmel *et al.*, 1998, Schilling and Kimmel, 1997). These anomalies were not observed at lower concentrations (5ug/ml) it only showed mild phenotype at 8-10 d.p.f. The embryos are viable beyond 10 d.p.f. 10 ug/ml prednisolone E3M produces readily scorable phenotype at 8-10 d.p.f. Viable beyond 10 d.p.f. 10ug/ml induced abnormalities of the spine with lateral curvature similar to a scoliosis and shown 58% viability 20 ug/ml prednisolone in E3M produces strong phenotype at 8-10 d.p.f. and shown 17% viability. At 40 ug/ml all the exposed embryos were dead after 48 hrs. The results showed that the prednisolone group at the concentration of 10 µg/ml can obviously use for development of model for osteoporosis. The skeletal anomalies were evaluated by using alizarin red dye at each alternate day exposure from 3dpf to 10dpf (Fig. 2). Mean raw integrated density (RID) was compared between 11 dpf control and GIOP model using different staining techniques Alizarin Red and Calcein live staining. n=21, two tailed paired t test, with groups having equal

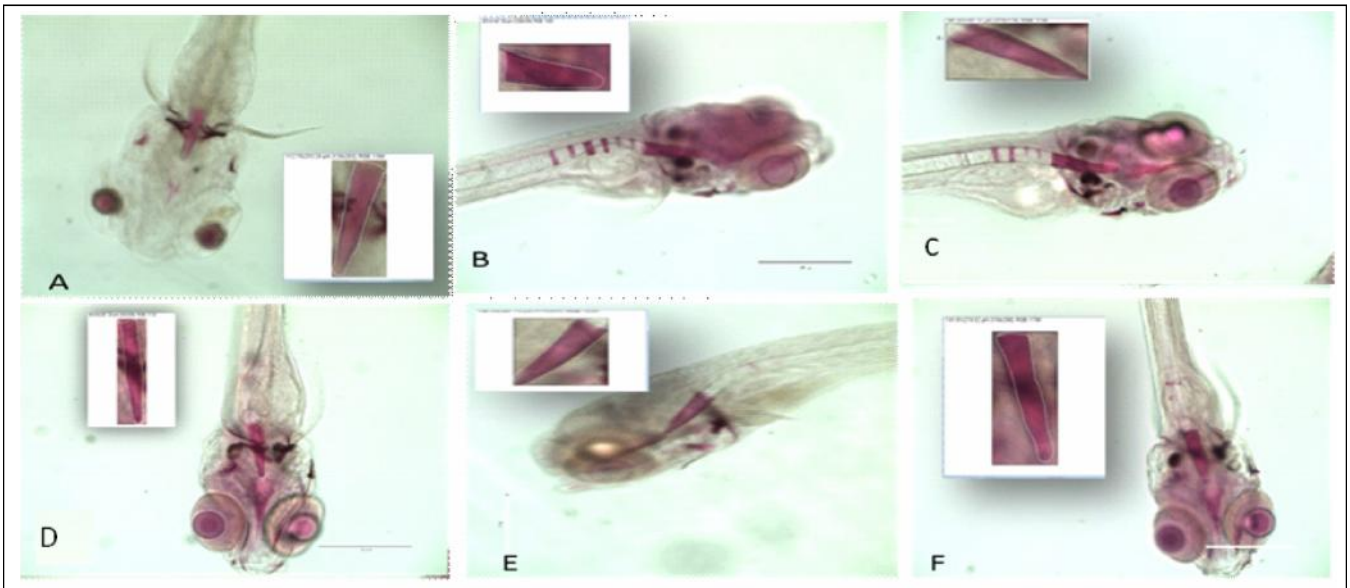

Fig. 2 : A,B,C showing images of Alizarin red stained 11dpf control and D, E, F - 10 ug/ml prednisolone treated larvae and zoomed area of the notochord used for used Image J analysis

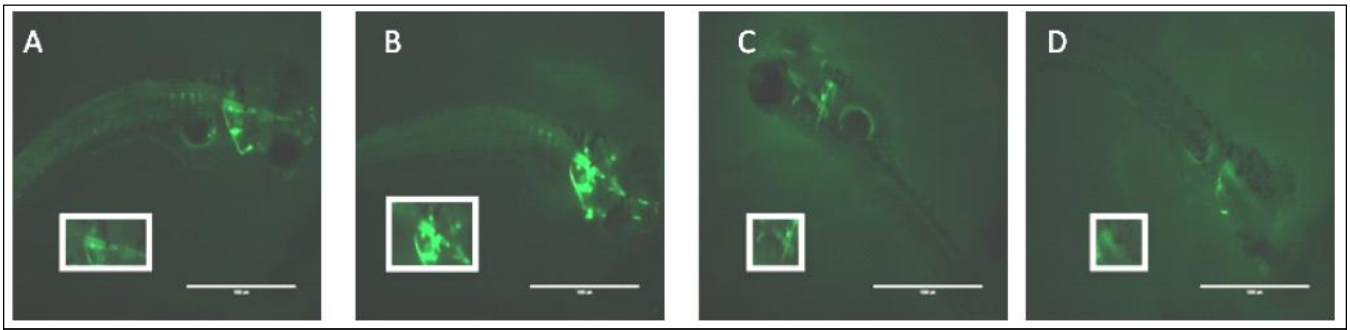

Fig. 3 : A, B showing images of Calcein stained 11dpf control and C, D- 10 ug/ml prednisolone treated larvae and zoomed area of the notochord used for used Image J analysis

variance, at 0.05 significance level shows that the means of control and GIOP model are statistically significantly at $p < 0.05$. Therefore, osteoporosis model using zebra fish induced by prednisolone was successfully developed.

The practice of homeopathy has great impact on human life. As such, there is a need to have a balanced investigation of the claims by its supporters and counter claims by its detractors to bring out the truth about the science behind Homeopathy. In addition, zebra fish osteoporosis model was successfully used to verify the effect of 3 homeopathy drug at different potencies (6 CH and 30CH) of *Calcarea carbonica*, *Argenticum metallicum* and *Sepia* in two dilutions. Mean raw integrated density compared at different time points 15 dpf, 25 dpf and 39 dpf control, GIOP untreated, GIOP treated larvae with Alendronate and homeopathic drugs using different staining techniques - Alizarin Red and Calcein live staining. n=21, two tailed paired t test, with group having equal variance, at 0.05 significance level. These were preliminary studies which showed that *Calcarea Carbonica 30c* and *Sepia 30* c show promising results on GIOP model for increased mineralization. Further studies are required in higher

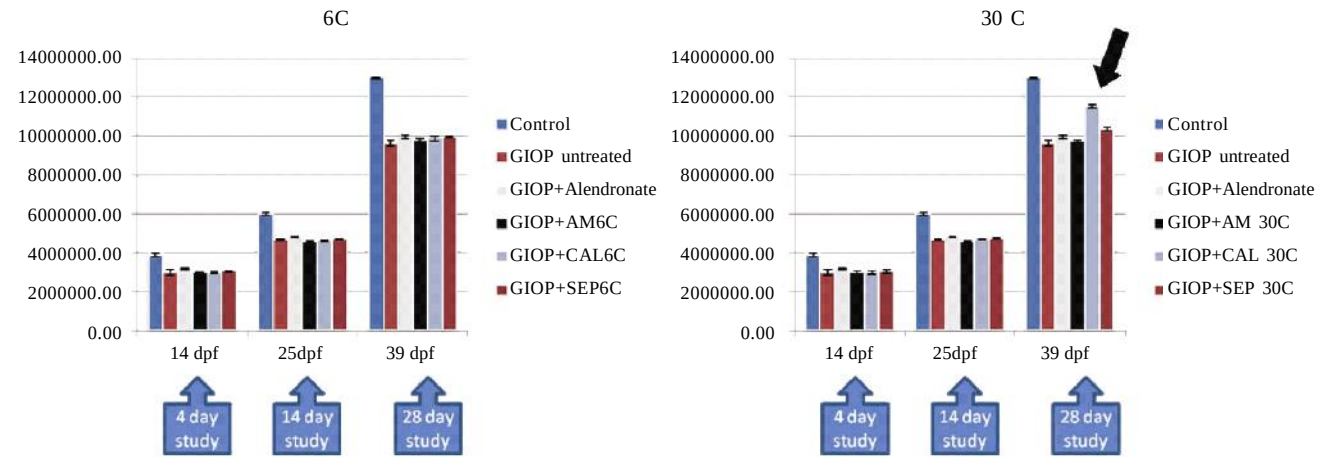

Fig. 4 : The means of control and GIOP are statistically significantly at $p < 0.05$. But the means of GIOP and other treated groups are not statistically significant at $p<0.05$ except in the case of 28 day study for *Calcarea Carbonica* 30C and *Sepia* 30C (marked with arrow) calculated by Alizarin red staining

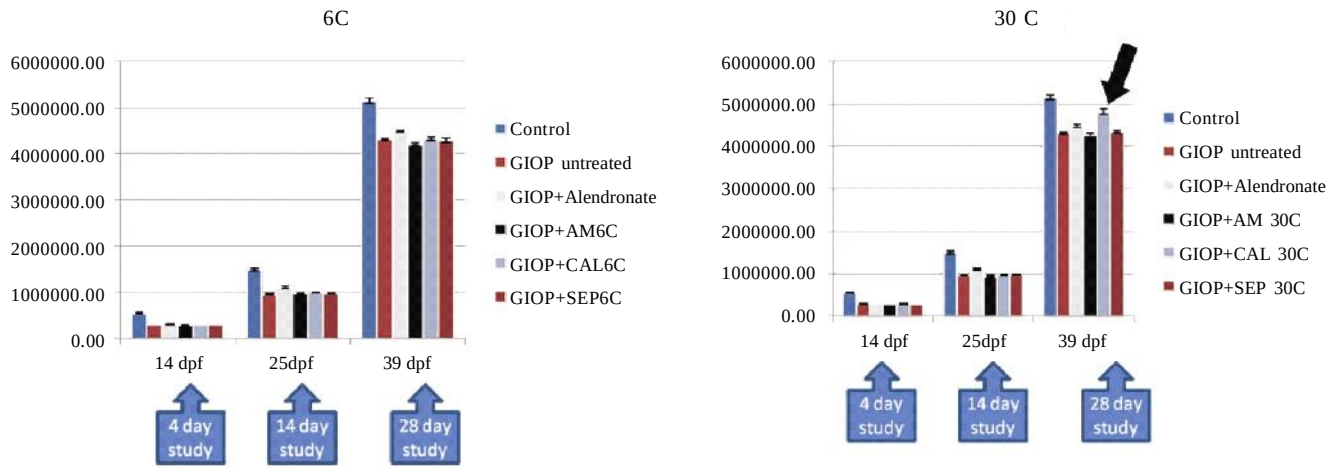

Fig. 5 : The means of control and GIOP are statistically significantly at $p < 0.05$. But the means of GIOP and other treated groups are not statistically significant at $p<0.05$ except in the case of 28 day study for *Calcarea Carbonica* 30C and *Sepia* 30C (marked with arrow) calculated by Alizarin red staining

vertebrate like rat to confirm the action.

Conclusion:

The role of homeopathy drugs are well known from ancient years. Homeopathy drugs are indicated for treatment of several diseases. However, in present day homeopathy practice, the synthesis procedures are often difficult to interpret from ancient texts. Different protocols exist to get several types of drugs for a single treatment. Hence, selection of the synthesis protocol requires sound knowledge of the homeopathy system. To address this issue, standardization, mechanism and its detailed documentation would be helpful. Standards for manufacture and quality control are not yet properly defined and enforced. Parallely, scientific studies on all the homeopathy drugs described in literature are needed. Pharmacological validation studies can be undertaken to generate evidence of the efficacy of these drugs. Further, detailed investigation of the mechanism of action using modern research tools (namely, proteomics and genomics) will help solve the mystery of the observed effects of these homeopathy medicines. So in the present study we deal with GIOP model in Zebra fish. Prednisolone, a Glucocorticoid was used as a model compound to develop a rapid and high efficient osteoporosis novel animal model.

A 6 day exposure of prednisolone solutions with a range of concentration starting from 5 dpf and continued for 6 days was carried out. Quantitative analysis of the stained area was performed by microscopic inspection and digital imaging methods to reflect the amount of bone mineralization using alizarin dye and live calcein labelling. The results showed that the prednisolone group at the concentration of 10 µg/ml can be used for inducing osteoporosis in zebrafish larvae. Scientific studies of three homeopathy drugs prescribed for osteoporosis were used to generate evidence of the efficacy of these drugs. Our results showed consistent pattern where the means of controls and GIOP were found to be statistically significantly at $p < 0.05$. But the means of GIOP and other treated groups at various time points (4, 14 and 28 days) were not statistically significant at $p<0.05$ except in the case of 28 days study for Calcarea Carbonica 30C and Sepia 30C using the mentioned staining techniques quantified using Image. Our results also demonstrate the importance of Zebrafish which can be potential screening and mechanism analysis platform for bone mineralization *in vivo*. It has the advantage of *in vitro* assays allowing for quick screening and affordability reliability for identifying the drug candidate, as also shown by our study formulations.

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