International Journal of Pharmaceutical Research and Applications
Volume 9, Issue 5 Sep - Oct 2024, pp: 469-478 www.ijprajournal.com ISSN: 2456-4494

Qualitative Analysis of Baclofen in In-Vitro experimental Model: a Repurposing Approach

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Date of Submission: 15-09-2024 Date of Acceptance: 25-09-2024

ABSTRACT

Pharmacology is a branch of modern medicine which deals with not only the actions and adverse effects of drugs but also involves in the identification of a new lead or drug candidate. In-vitro pharmacology explicit the cellular and molecular levels of biological organization and the delineate the drug actions thereof. The drug discovery is a much complicated, long time and huge money consuming process. Repurposing is a wise full choice to identify new drug leads as well as reduce the time and money consumption of drug discovery process. In this project work we have selected baclofen for the sake of repurposing. Baclofen was systematically studied for its in-vitro anti-muscarinic actions by using chicken ileum. We have evaluated the anti-muscarinic actions of the drug by applying various quantifying techniques of bio-assay. The systematic approach in invitro studies have been adopted in this experimental study with standard environmental and laboratory conditions. The experiments were performed in duplicate simultaneously to minimize the chance of manual errors and practical bias. The study result showed that, baclofenhaspronounced anti-muscarinic actions with an IC-50 value of 1.6 respectively. This finding hints the possibilities of analysing the drugfor further in-vivo and extensive pharmacological screening for clinical anti-cholinergic use. The varied draw backs of existing anti-muscarinic drugs shall be put aside if the test drug will succeed in its goal. We suggest further pharmacological screening of thedrug in future.

Keywords: Receptor pharmacology, Repurposing, Invitro systems, Antimuscarinic, Antagonist, Drug efficacy, Experimental pharmacology Half maximal inhibitory concentration (IC50), muscarinic actions, new lead.

I. INTRODUCTION

Developing a pharmaceutical is a highly intricate process involving multiple rounds of assays and tests. Throughout its development, from screening potential molecules to identifying the lead compound and conducting pre-clinical trials, ensuring efficacy and safety is paramount. In vitro pharmacology plays a crucial role in this process, allowing researchers to gather valuable data on the effects of drug candidates outside of living organisms. This involves conducting studies and experiments on microorganisms and cells in environments that differ from their natural biological settings. Thus, in vitro pharmacology entails the examination of the biological impacts of drugs and pharmaceuticals in settings external to living organisms [1,2]. Experimental animals such as rats, guinea pigs, and rabbits are commonly utilized for biological assays. Utilizing ex vivo models for pharmacological experimentation presents a viable solution. These models replicate laboratory conditions accurately without necessitating the sacrifice of live animals. By adopting ex vivo models, researchers can conduct experiments on isolated tissue preparations effectively, mimicking real-world laboratory conditions while prioritizing ethical considerations and reducing reliance on live animal subjects for educational purposes [3,4]. Isolated chicken ileum and duodenum preparation were most widely used in experiments of Pharmacology subject and used for the determination of pA2 value, three-point/four- point bioassay, determination of effect of an antagonist on DRC of a drug, and comparison of activities of different agonists like acetylcholine and barium chloride.[5]

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Volume 9, Issue 5 Sep - Oct 2024, pp: 469-478 www.ijprajournal.com ISSN: 2456-4494

Isolated tissue bath techniques facilitate the examination of various steps in pharmacological studies, such as radioligand binding to assess drug affinity and measuring second messengers. Maintaining tissue function allows for the calculation of pharmacological variables that hold greater significance within a tissue context compared to cellular studies. This approach better approximates how drugs would function within the entirety of the body, enhancing the relevance and applicability of experimental findings to real-world scenarios [6].

In a bioassay, a stimulus is administered to a subject, leading to a measurable change in the subject's characteristics. The extent of this change varies depending on the dosage of the stimulus [7]. During the preclinical evaluation of a new compound, its biological activity is compared to that of a known standard compound using suitable test systems. Biological assays are employed to assess the relative potency of these two preparations, typically comparing a standard with an unknown compound [8].

Cholinergic muscarinic receptors are G protein-coupled (metabotropic) receptors and the nicotinic receptor is ion channel (ionotropic) receptors. Pharmacologic molecular cloning studies have led to the classification of muscarinic acetylcholine receptors in central and peripheral tissues into five distinct muscarinic receptor subtypes—M1,M2, M3, M4, and M5 [9].

II. MATERIALS AND METHODS

Materials

Chicken ileum, Acetylcholine, Baclofen tablet, Physiological salt solution

1.Collection of chicken ileum

The freshly isolated gastrointestinal tract of a healthy cock (Gallus gallus, family Phasianidae) was obtained from a local slaughterhouse, transported in an ample volume of chick ileum solution, and kept aerated during transit to the laboratory. The caecum was lifted forward and the ileo-caecal junction was identified. The ileum was cut and transferred to a dish containing Tyrode solution.[16]

2.Drug solutions

a) Preparation of physiological salt solution

The physiological salt solutions are used to keep isolated tissue or organs up to the experimental duration. Physiological solution is important for the survival of tissue ex vivo. Here we used Modified Tyrode (chick ileum) solution as the physiological salt solution because our tissue preparation is chicken ileum [10]The required amount of modified Tyrode solution (chick ileum solution) is prepared using the following compounds [11].

Table1e 1 PSS Composition table

Reagents	**Quantity**
Sodium chloride	69.19 gm
Potassium chloride	3.42 gm
Calcium chloride	2 2.94 gm
Dextrose	65.17gm
Magnesium Chloride	2 1.01 gm
KH2PO4	1.63 gm
NaHCO3	21.01gm

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It is prepared carefully using analytical-grade reagents and distilled water. The other precautions to be taken are adjusting the pH of the final solution and aeration with oxygen, the mixture of oxygen and carbohydrate (95%+5%), or even bubbling with air. The physiological solution thus prepared should be clear, and if turbid, it is recommended to make a new solution before commencing the experiment.[12]

b) Drugs and Chemicals

Acetylcholine and Baclofen were used in the study. The other chemicals and reagents used were of analytical grade. Stock solution of acetylcholine was prepared in (1000µg/ml) concentrations by using distilled water. Similarly, baclofen solutions were prepared using distilled water and then diluted to the desired concentration with physiological salt solution. For smooth muscle preparation, only freshly prepared Tyrode solution (pH 7.3 to 7.4) was utilized.[13]

Molecular weight of Baclofen = 213.661g/mol

Standard drug solution of Baclofen

500 µmol/ml was prepared by dissolving 0.1mg of Baclofen in a physiological salt solution made up to the volume of 500ml. [14]

III. METHODOLOGY

1.Preparation and mounting of tissue for experiment

A three-centimetres section of ileum was cleaned, cut, and cleared of intestinal contents and mesenteric attachments. Threads were tied at both ends, ensuring the lumen remained open. The tissue was then mounted in an organ bath containing Tyrode solution, with the temperature maintained at 34 ± 1°C, and allowed to equilibrate for 30 minutes. A load of 0.5 g was applied, with a magnification of 5-7 times, and a bath volume of approximately 15 ml. The preparation was washed with Tyrode solution every 10 minutes. The drum speed adjusted to 2.5 rpm. Aeration must be provided to the tissue preparation. One end of the tied ileum was attached to the aeration tube and the other to the frontal writing lever. The tip of the lever contains ink for writing in the drum. The time cycle is repeated (baseline 30sec, contact time 60sec, 1min for 3 washing).[17]

2. Experimental protocol for Baclofen

2.1 Effect of Baclofen on CRC of Acetylcholine

After mounting the tissue, establish the baseline approximately 2-3 cm below the one-third mark of the drum. Add 0.1 ml of the drug solution (Ach) and record the drug's response for 60 seconds. Rotate the drum and wash the tissue with Tyrode solution 2-3 times, allowing a rest period of at least 30 to 60 seconds between each wash. Record the response at increasing order of doses (0.1,0.2,0.4,0.8 and 1.6ml) of acetylcholine to a maximum followed by 1 min gap then repeat the procedure with Baclofen test solution at increasing order of doses, which contain 500µmol/ml of drug. Properly label the graph.[18]

2.2 Antagonism (Identification of IC50 and Dose ratio).

2.2.1 Incubation with Baclofen for up to one minute.

The dose-response curve (DRC) of acetylcholine (Ach) was constructed with a contact time of 60 seconds. Contractions were recorded using an isotonic lever on a kymograph at a speed of 0.25 rpm. Ach responses at concentrations of 100 µg/ml (0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml, and 1.6 ml) were documented. After recording the response at each concentration, the tissue was washed three times with physiological salt solution at 1-minute intervals. Responses were recorded in several phases: baseline recording (0-30 s), contact time (30-60 s), first wash (60-120 s), second wash (120-180 s), and third wash (180-240 s). Contractions were measured as changes in height from the baseline and expressed as a percentage of the maximum response. [7]

The response to acetylcholine was recorded until the maximum dose was achieved, and from this data, the IC50 value of acetylcholine before baclofen treatment was calculated. The tissue was then washed with physiological salt solution and incubated with 500 µMol/ml concentration of the antagonist (Baclofen) for 1 minute. A new concentration-response curve was established in the presence of 500 µMol/ml Baclofen dissolved in the physiological salt solution. Contractions were recorded in the same manner as the initial CRC. The height of the contractions was measured from these graphs and converted into percentage responses. From this data, the IC50 value of acetylcholine after baclofen treatment was calculated. [19]

2.2.2 Incubation with Baclofen up to 2 minutes

The dose-response curve (DRC) of acetylcholine (Ach) was constructed with a contact time of 60 seconds. Contractions were recorded using an isotonic lever on a kymograph at a speed of 0.25 rpm. Responses were documented for acetylcholine at a concentration of 100 µg/ml,

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using volumes of 0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml, and 1.6 ml. After recording each response, the tissue was washed three times with physiological salt solution at 1-minute intervals.

The time cycle for this preparation included baseline recording (0-30 seconds), acetylcholine response contact time (30-60 seconds), first wash (60-120 seconds), second wash (120-180 seconds), and third wash (180-240 seconds). Responses to acetylcholine were measured as changes in height from baseline and expressed as a percentage of the maximum response. The response was recorded until the maximum dose was achieved, from which the IC50 value of acetylcholine before baclofen treatment was calculated.

The tissue was then washed with physiological salt solution and incubated with 500 µMol/ml of the antagonist Baclofen for 2 minutes. A new concentration-response curve was then established in the presence of 500 µMol/ml Baclofen dissolved in physiological salt solution. Contractions were recorded in the same manner as the initial dose-response curve. The heights of the contractions were measured from these graphs and converted into percentage responses, allowing for the calculation of the IC50 value of acetylcholine after baclofen treatment [19].

2.2.3 Maintaining a similar concentration of baclofen as that of acetylcholine

CRC of Ach(100µg/ml) was taken by using 0.1ml,0.2ml,0.4ml,0.8ml&1.6ml followed by 3 successive washing with Tyrode solution. In the same way CRC of baclofen was recorded using the same concentration that of Ach (100µg/ml baclofen). Then inject baclofen(0.1ml) provide contact time for 30 secs followed by same dose of Ach (0.1ml). In the same manner CRC is recorded by using various concentrations such as 0.1ml, 0.2ml. 0.4ml, 0.8ml & 1.6ml of Ach and baclofen.[20]

2.2.4 Successive antagonist evaluation of Baclofen with varying concentration

Record CRC of acetylcholine by using 0.1ml,0.2ml,0.4ml, and 0.8ml. Select two suitable responses (0.2ml,0.8ml) and repeat the selected doses, along with the same dose of acetylcholine within the presence of baclofen. compare the relative response.[21]

2.2.5 Cumulative antagonism with Baclofen

Cumulative dose-response curves with an agonist are recorded alternatively in the absence and presence of different concentrations of an antagonist. For this record, various concentrations of acetylcholine agonists are in increasing order without taking baseline and washout. Gave a maximum of 30-60 seconds of contact time for each dose. In the same way, record the cumulative response of antagonist baclofen.[22]

IV. RESULTS

I. Effect of Baclofen on CRC of Acetylcholine

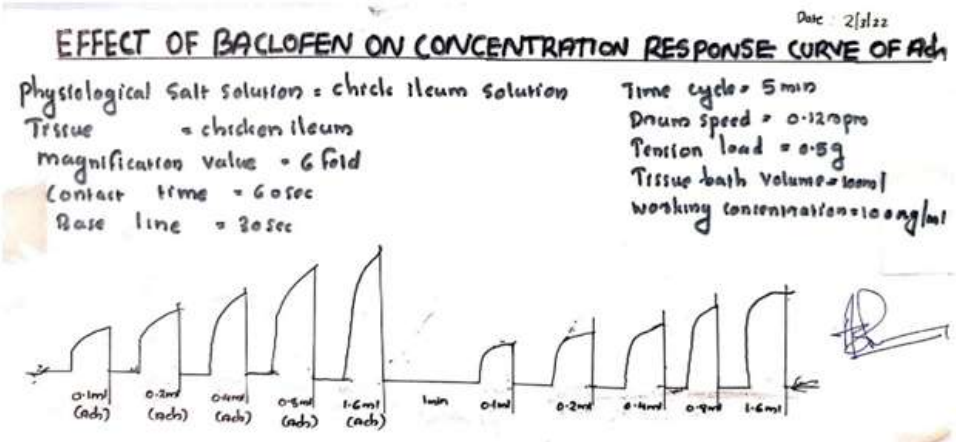

Figure No 1. Effect of Baclofen on CRC of Acetylcholine

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II. Antagonism (identification of ic_{50} and dose ratio)

1) Incubation with Baclofen for up to one minute.

Plot the graph by taking concentration on X the axis and percentage response on Y- the axis. IC50 calculated from this graph. IC50 is the concentration (or dose) effective inproducing 50% of the maximal response and is a convenient way of comparing drug potencies

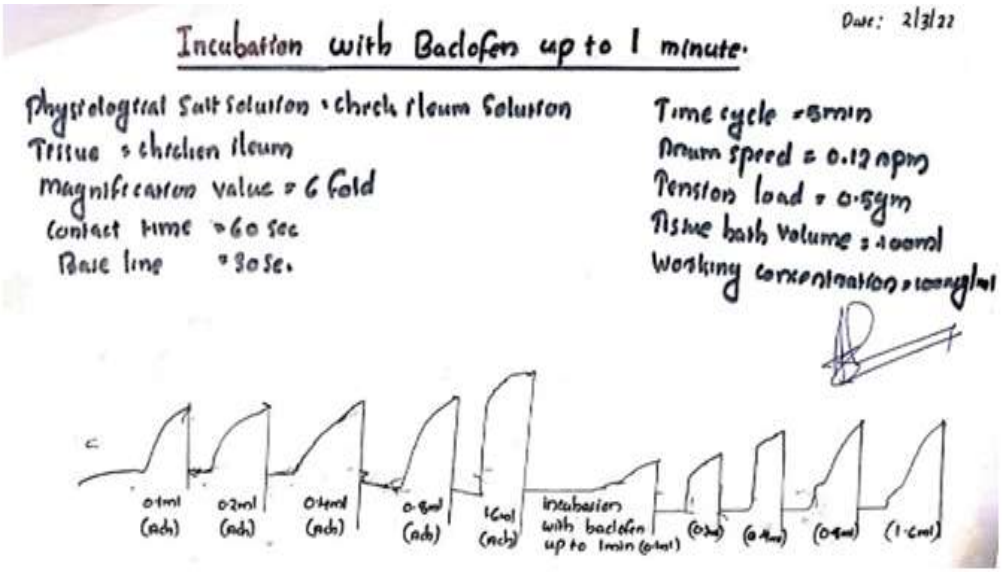

Figure No 2. Incubation with baclofen for up to one minute

Table2: Observation table for recording height of response (Acetylcholine and Acetylcholine + baclofen for one minute)

Drugs	Volume	Height of Response	Percentage of response
Ach (100µg/ml)	0.1ml	1.8cm	55%
	0.2ml	2cm	61%
	0.4ml	2.3cm	70%
	0.8ml	2.8cm	85%
	1.6ml	3.3cm	100%
Ach(100µg/ml) + Baclofen(500µmol/ml	0.1ml	1cm	36%
	0.2ml	1.5cm	54%
	0.4ml	2cm	71%
	0.8ml	2.3cm	82%
	1.6ml	2.8cm	100%

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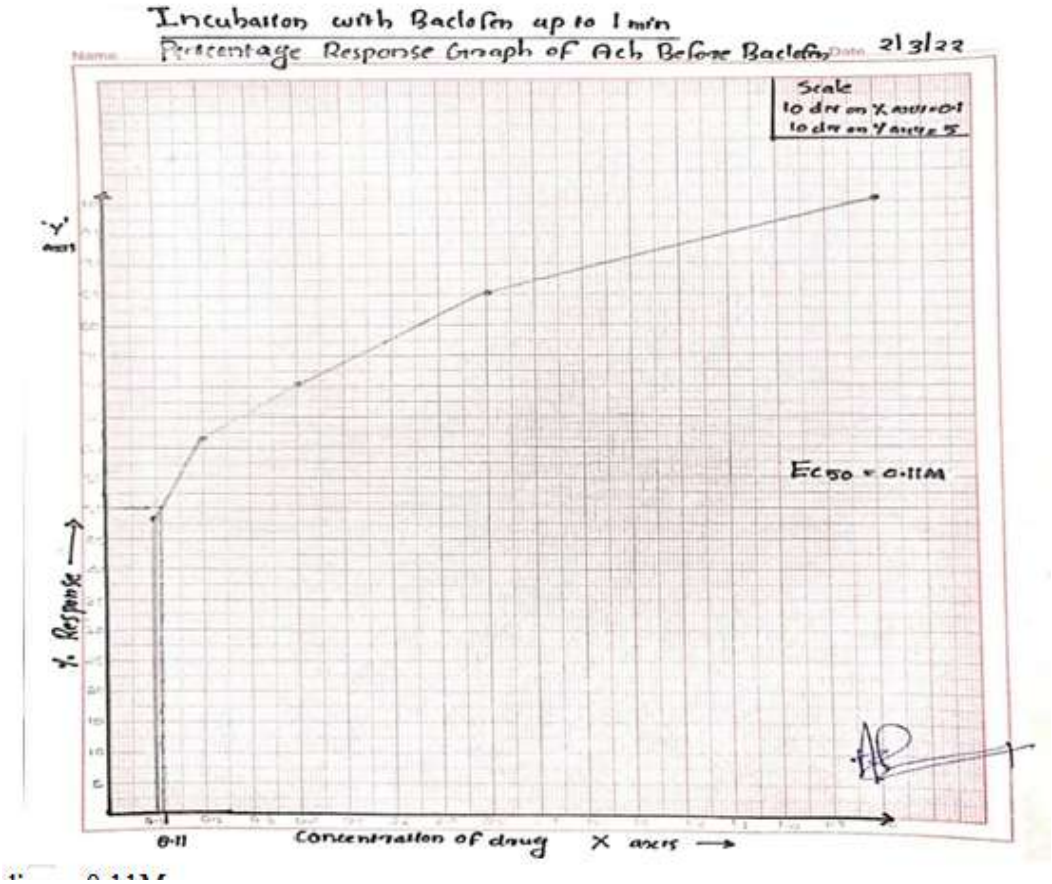

Figure No.3 IC 50 of baclofen

IC_{50} of acetylcholine = 0.11M
IC_{50} of acetylcholine after treatment with baclofen = 0.18M
DOSE RATIO = IC_{50} of acetylcholine after baclofen / IC_{50} of acetylcholine before baclofen
= 0.18/0.11

2)Incubation with Baclofen for up to two minutes

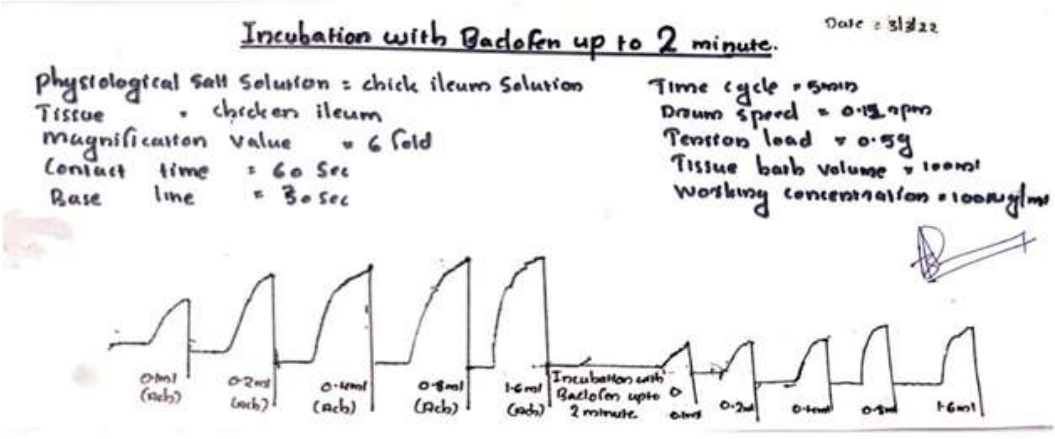

Figure No.4Incubation with baclofen for up to two minutes

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Plot the graph by taking concentration on X the axis and percentage response on Y- the axis. IC50 is calculated from this graph. IC50 is the concentration (or dose) effective in producing 50% of the maximal response and is a convenient way of comparing drug potencies.

Table 3: Observation table for recording height of response (Acetylcholine and Acetylcholine + baclofen for two minutes)

Drugs	Volume(ml)	Height of response	Percentage of drug
Ach(100µg/ml)	0.1ml	1.7cm	47%
	0.2ml	2.4cm	67%
	0.4ml	3.1cm	86%
	0.8ml	3.4cm	94%
	1.6ml	3.6cm	100%
Acetylcholine (100µg/ml) +Baclofen (500µmol/ml)	0.1ml	0.7cm	35%
	0.2ml	0.9cm	45%
	0.4ml	1.3cm	65%
	0.8ml	1.8cm	90%
	1.6ml	2cm	100%

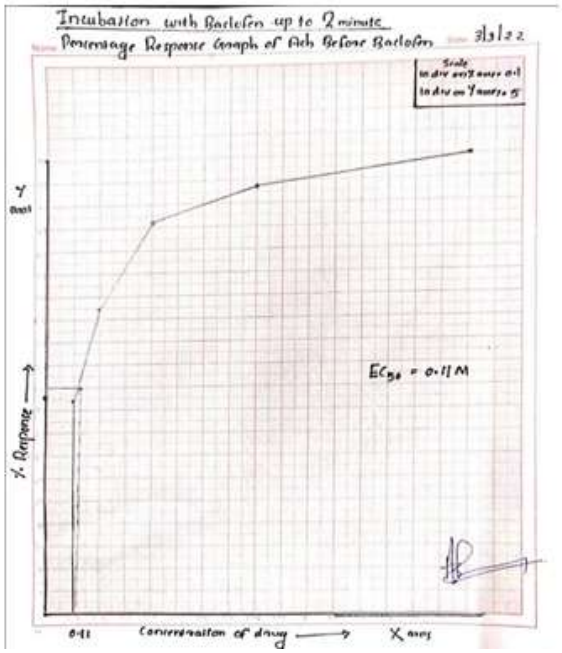

Figure No 5. Graph represents IC50 value of Baclofen (2 minutes incubation)

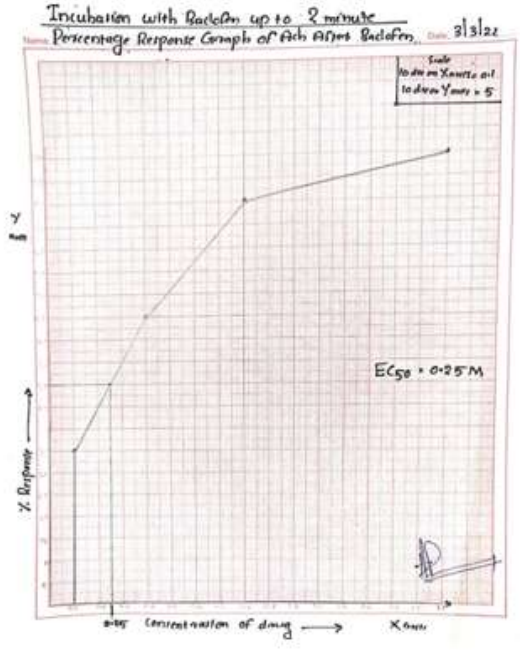

Figure No 6. Graph represents IC 50 value of baclofen (2 minutes incubation)

IC_{50} Of acetylcholine before baclofen =0.11ml
IC_{50} OF acetylcholine after baclofen =0.25ml
DOSE RATIO =IC_{50} of acetylcholine after baclofen/ IC_{50} of acetylcholine before baclofen
= 0.25/0.11
= 2.27

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3) successive antagonist evaluation of baclofen with varying concentration

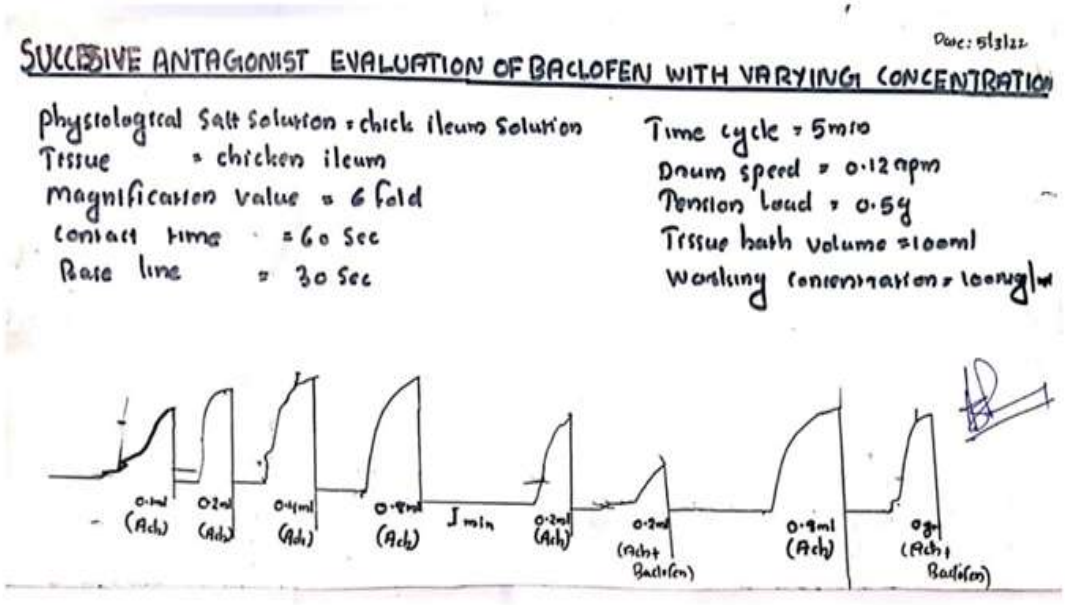

fig 7.successive antagonist evaluation with Baclofen

4)Maintaining similar concentration of baclofen and acetylcholine

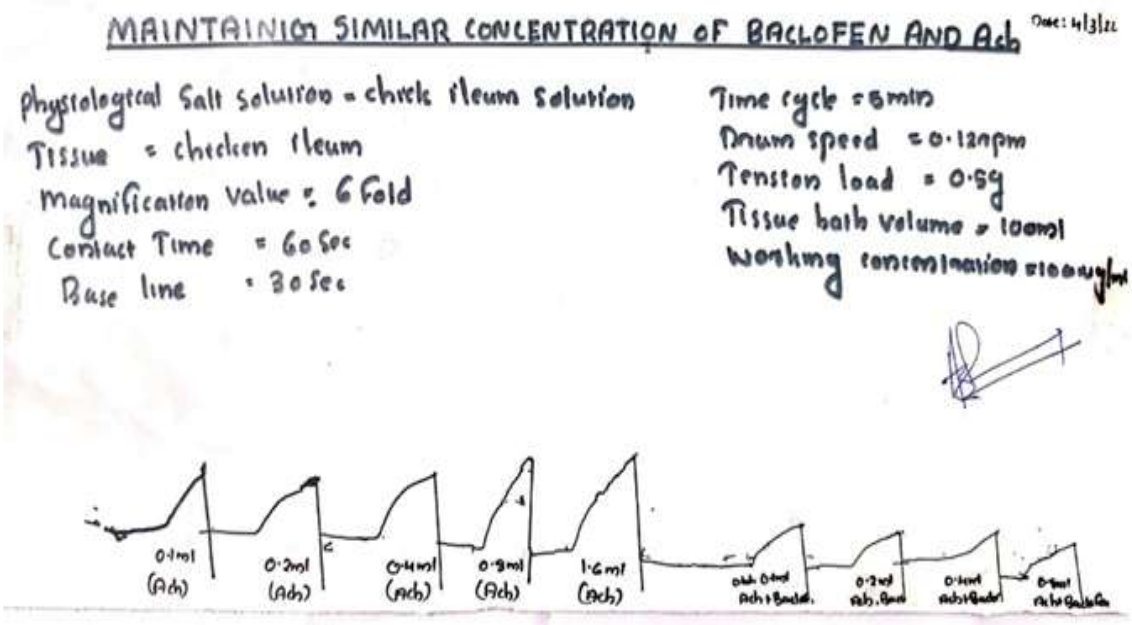

Fig 8. maintaining similar concentration

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5) Cumulative antagonism with Baclofen

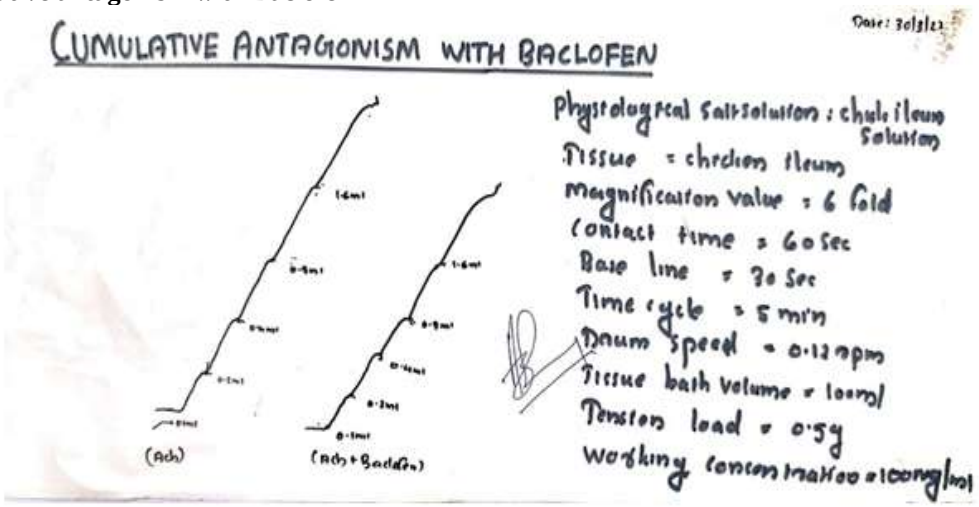

Fig 9.cumulative antagonism with Baclofen

V. DISCUSSION

The in-vitro pharmacology is the core of systematic biology which identifies the basic mode of action of a drug. In this study, we have considered an approach of repurposing to identify a better anti-muscarinic agent. Anti-muscarinic agents have a wide range of clinical application including spasmolytic, anti-secretory, anti-asthmatic, anti-Parkinson's, etc…The need for search of a novel anti-muscarinic drug already explained in the objective section. The effect of baclofen in CRC of acetylcholine showed a pronounced decrease in the height of response. This states that baclofen could have an anti-cholinergic action by possibly acting on either M3 and other muscarinic receptors. This indicates that baclofen has a possible anti-cholinergic action on a cellular and receptor level. Further we have studied the effect of baclofen on CRC of acetylcholine and identified the IC-50 value along dose ratio. The dose ratio of baclofen was found to be 1.6. With similar experiments conditions, the dose ratio of atropine was found to be 0.72 in our laboratory. Further, after plotting CRC with acetylcholine we have incubated the tissue with 500µmol of baclofen in the suitable volume as described in procedure. Later the same procedure is repeated same procedure in another tissue bath with two minutes incubation. These results showed that baclofen as another anti-cholinergic has the ability to occupy the receptor in time-dependent manner.

In another experiment, we have maintained the concentration of acetylcholine and baclofen in similar and CRC was plotted. Interestingly the baclofen-treated tissue has a pronounced decrease in height of response when comparing acetylcholine alone. Later, successive evaluation of baclofen antagonism by selecting two responses from a CRC of acetylcholine was carried out. There was a pronounced decrease in the height of response when comparing the acetylcholine-induced contractions. In cumulative antagonism, the cumulative dose-response of acetylcholine was plotted first, then the cumulative CRC was repeated in the presence of baclofen and found a pronounced decrease in the height of response.

The in-vitro pharmacology is the core of systematic biology which identifies the basic mode of action of a drug. In this study, we have considered an approach of repurposing to identify a better anti-muscarinic agent. Anti-muscarinic agents have a wide range of clinical applications including spasmolytic, anti-secretory, anti-asthmatic, anti-Parkinson's, etc…The need for search of a novel anti-muscarinic drug already explained in objective section. The effect of baclofen in CRC of acetylcholine showed a pronounced decrease in the height of response. This states that baclofen could have an anti-cholinergic action by possibly acting on either M3 and other muscarinic receptors. This indicates that baclofen has a possible anti-cholinergic action on a cellular and receptor level. These results showed that baclofen as other anti-cholinergic has the ability to occupy the receptor in time time-dependent manner.

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VI. CONCLUSION

From our experiment study, we have identified the anticholinergic actions of baclofen in chicken ileum by using standard protocols. The results have given a strong indication about the test drug is to be considered as anti-cholinergic agent in the in-vitro system. We propose a further pharmacological evaluation of this existing drug to be used as a clinical anti-cholinergic drug in the future.

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