Development of an *ex vivo* model for pharmacological experimentation on isolated tissue preparation

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Abstract

Pharmacology as a subject depends largely on experiments conducted in laboratory animals. Experimental animals like rat, guinea pig, rabbit, etc. are used for the biological assay. For the teaching purposes to use isolated strip preparations from various organs, the laboratory animal species has to be sacrificed just for a piece of tissue. The present study was aimed to develop *ex vivo* model for pharmacological experimentation, which will mimic the actual laboratory condition without sacrificing the experimental animals. Dose response curve of acetylcholine alone and in presence of different concentrations of atropine was plotted using isolated chicken ileum, chicken duodenum, rat ileum, and rat duodenum and their EC\(_{50}\) values were compared. The effect of atropine in terms of its type of antagonism was predicted based on Schild plot and pA\(_2\) values were obtained. The chicken ileum and duodenum were also evaluated for four- and three-point bioassay, respectively. The results suggested that acetylcholine produced a dose-dependent increase in contraction in both chicken and rat ileum and duodenum preparation. The concentration response curve of acetylcholine in chicken ileum shifted toward left side of rat ileum with a higher EC\(_{50}\) value. Atropine shifted the concentration response curve of acetylcholine toward right with a change in EC\(_{50}\) value. Schild plots indicated that antagonism produced by atropine was found to be competitive in nature. The pA\(_2\) values of atropine were found significantly high with isolated chicken ileum as compared to rat ileum preparation. It is concluded that isolated chicken ileum and duodenum preparation can be employed for routine experiments of pharmacology subject and the use of these isolated preparations is a novel approach for managing pharmacological experiments and importantly, without sacrificing the experimental animals.

Key words: Chicken ileum, duodenum, four point bioassay, pA\(_2\) value, three point bioassay

INTRODUCTION

The experimentation in pharmacology largely depends on laboratory animals. Experimentation on animals in laboratories generally falls into one of the following three categories: toxicity testing, education and training, and basic or applied research. Mice are preferred species in pharmacological experimentation because of their small size, ease of breeding, and short generation time. Rats, guinea pigs, rabbits, and dogs are also used; each has special characteristics that make it optimal for certain types of tests.\(^1\), \(^2\) Research, in reality, involves three facets: acquisition of new knowledge, use of animals in teaching exercises, and the testing of compounds, chemicals, or devices for safety and effectiveness. There must be reasonable expectation that research involving animals will contribute significantly to present and future knowledge which may eventually lead to the protection and improvement of the health and welfare of either human beings or animals. Maintaining animals under healthy conditions is an essential pre-requisite for animal experimentation. The Committee for the Purpose of Control and Supervision of Experiments on Animals

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(CPCSEA) was appointed as a watchdog (pardon the pun) to oversee that laboratory animals were well maintained and that the experiments which were conducted on them were done according to internationally accepted ethical norms. The ultimate goal of any animal welfare measure is the elimination of all experiments on animals that are likely to cause pain or distress.[3] But this can be only a dream, so the next measure is to think of strategies that can eliminate/replace the use of animals or at least reduce the number as much as possible.[4]

Quantitative experiments are designed to analyze and assay the activity because of the need to assay useful drugs which could not be measured by any physical or chemical methods. Estimation of the potency of an active principle in a unit quantity of preparation or detection and measurement of the concentration of the substance in a preparation using biological methods (that is observation of pharmacological effects of living tissue micro/macros or immune cells or animal) is known as biological assay or bioassay.[5] The basic principal of bioassay is to compare the test substance with the International Standard Preparation of the same and to find out how much test substance is required to produce the same biological effect, as produced by the standard.[6] Bioassay can be performed either by interpolation, matching, bracketing, three-point assay, and four-point assay. Experimental animals like rat, guinea pig, rabbit, etc. are used for assay.[7, 8] For the teaching purposes to use isolated strip preparations from various organs, the laboratory animal species has to be sacrificed just for a piece of tissue. However, chicken ileum is a tissue that is easily available from the slaughterhouse and animals need not be killed additionally for experimental purpose. This might reduce, refine, and replace the number of animals to be used for teaching purpose.

The aim of the proposed work is to find out an alternative to the animal experimentation that will mimic the animal laboratory condition from living biological tissues which are otherwise wasted daily. The present work aims to investigate the suitability of chicken ileum and chicken duodenum for ex vivo determination of effects of drugs upon the tone and contractility of the intestinal strip in comparison with rat ileum and rat duodenum using various bioassay methods.

MATERIALS AND METHODS

Drugs and Chemicals

Acetylcholine chloride and atropine sulfate used in the study were obtained from Loba Chemie, Mumbai, India. The other chemicals and reagents used were of analytical grade. Stock solution of acetylcholine was prepared in 5% (w/v) NaH₂PO₄ solution so as to contain 1 mg/ml[7] various concentrations were prepared using distilled water. In the same way solutions for atropine were prepared in distilled water and diluted to appropriate concentration with physiological salt solution. Only freshly prepared Tyrode solution (pH 7.3 to 7.4) was used for smooth muscle preparation.

Animals

Wistar rats of either sex weighing 120- to 180-g were used. Animals were housed under standard conditions of temperature (25 ± 2 °C), 12 h/12 h light/dark cycle, and fed with standard pellet diet and water ad libitum. The animals were allowed to acclimatize for one week before the experiments. All experimental protocols were approved by the Institutional Animal Ethics Committee constituted as per the Committee for the Purpose of Control and Supervision on Experiments on Animals (IAEC proposal no. 06/2009, CPCSEA registration no. 994/a/GO/06/CPCSEA/23/Oct/2006). Fresh entire gastrointestinal tract of healthy cock was obtained from a local slaughterhouse.

Smooth muscle preparation and assembly

Fresh entire gastrointestinal tract of healthy cock (Gallus gallus domesticus, family Phasianidae) was obtained from a local slaughterhouse and was transported in 200-ml Tyrode solution to the laboratory. Aeration was provided immediately in the laboratory. The caecum was lifted forward and the ileocaecal junction was identified. The ileum and duodenum were cut and transferred to a dish containing Tyrode solution, 2-cm portion of both ileum and duodenum was cut, and intestinal contents were removed and freed from mesenteric attachments. A thread was tied at each end taking care that tissue is left open and the thread does not close the lumen. Tissue was mounted in an organ bath containing Tyrode. The temperature was maintained at 34 ± 1°C and allowed to equilibrate for 30 minutes. Load was adjusted to 0.5-g, the magnification from 5-7 folds and bath volume of about 15-ml was maintained. The preparation was washed every 10 minutes with Tyrode.[6] The same procedure was applied for rat ileum and rat duodenum muscle preparation also.

Dose Response Curve of Acetylcholine Alone and in Presence of Different Concentrations of Atropine and Comparison of Their EC₅₀ Values

Dose response curve (DRC) to acetylcholine was constructed with a contact time of 60 seconds. Contractions were recorded using isotonic lever on kymograph with speed of 0.25 mm/s. Responses to different molar concentrations of acetylcholine (1.14 × 10⁻⁶ to 2.337 × 10⁻⁴) were recorded as changes in height from baseline and expressed as percent of maximum response of the acetylcholine. After recording the response with a particular concentration of the agonist, the tissue was washed thrice with physiological salt solution at an interval of 1 minute. The time cycle for this preparation were as, base-line recording (0-30 s), response of
acetylcholine that is contact time (30-60 s), first wash (60-120 s), second wash (120-180 s), and third wash (180-240 s). The response of acetylcholine was taken till the ceiling dose was achieved. From this, EC<sub>50</sub> value of acetylcholine was calculated.\cite{9}

Once ceiling effect was observed and confirmed with the next higher dose, the tissue was washed with physiological salt solution and incubated with 3.45 -nM concentration of the antagonist (atropine) for half an hour and again concentration response curve was established in the presence of 3.45 -nM concentration of the antagonist being dissolved in the physiological salt solution. Like this contractions were recorded in the same manner for 11.40 and 34.55 -nM concentration of the antagonist. Thus, from these graphs, height of contractions were measured and converted into percentage responses with respect to the maximum contraction of the agonist alone, being assigned cent percent. Following this mean, standard error of mean (SEM) of the percentage response and log molar concentrations of the dose of the acetylcholine were obtained.\cite{10, 11} This procedure was repeated with chicken ileum and duodenum and rat ileum and duodenum muscle preparation.

Finally, the effect of atropine in terms of its type of antagonism was predicted based on Schild plot and pA<sub>2</sub> values were obtained. The chicken ileum and duodenum were also evaluated for four- and three-point bioassay, respectively.\cite{10, 11}

### Statistical Analysis and Calculations

Values are expressed as mean ± SEM. Mean concentration response curve to contraction rate were analyzed by the equation (given below) using non-linear regression of GraphPad Prism software (GraphPad Software Inc., San Diego, USA).

\[ Y = \text{Bottom} + \left(\left(\text{Top} - \text{Bottom}\right)/\left(1 + 10^{\left(\text{EC}_{50} - X\right)}\right)^p\right) \]

Where, X is logarithm of molar concentration of the relaxant, Y is the response produced by the agonist, P is the slope of the DRCs, EC<sub>50</sub> is the concentration (M) of the relaxant that produces 50% of its maximum response, and Agonist dose ratios (dr) were determined from the EC<sub>50</sub> on the DRCs with or without antagonists. The plot of log [dr – 1] \text{log} [antagonist] was analyzed by linear regression. Antagonism was considered to be competitive if the slope (m) of regression line was not significantly different from unity. In cases, a mean pA<sub>2</sub> value was obtained from individual estimated using the equation.

\[ \text{pA}_2 = \log (\text{dr}-1) - \log [\text{antagonist}] \]

Potency of drug using four- point bioassay methods on chicken ileum was calculated using following formula:

\[ \text{Potency} = n_1/t_1 \text{antilog}\left[\left(T_2-S_2\right)+\left(T_1-S_1\right)/\left(T_2-T_1\right)\right] \times \log n_2/n_1 \]

Where, \( n_1 \) = lower standard dose, \( n_2 \) = higher standard dose, \( t_1 \) = lower volume of test, \( t_2 \) = higher volume of test, \( S_1 \) = mean response of \( n_2 \), \( S_2 \) = mean response of \( n_2 \), \( T_1 \) = mean response of \( t_1 \) and \( T_2 \) = mean response of \( t_2 \).

Potency of drug using three- point bioassay methods on chicken duodenum was calculated using following formula:

\[ \text{Potency} = n_1/t \text{antilog}\left[\left(T-S_2\right)/\left(S_2\right)\right] \times \log n_2/n_1 \times \text{Cs} \]

Where, \( n_1 \) = lower standard dose, \( n_2 \) = higher standard dose, \( t \) = test dose (ml), \( S_1 \) = response of \( n_2 \), \( S_2 \) = response of \( n_2 \), T = response of test dose, and Cs = concentration of standard.

### RESULTS

#### Effect of Acetylcholine on Isolated Chicken Ileum, Chicken Duodenum and its Comparison with Rat Ileum and Rat Duodenum

It was found that acetylcholine produced a dose-dependent increase in contraction in both chicken and rat ileum and duodenum preparation. These results suggest that acetylcholine induce contractility of the intestinal strip. The concentration response curve of acetylcholine in chicken ileum shifted toward left side of rat ileum [Figure 1] with a higher EC<sub>50</sub> value [Table 1].

#### Effect of Atropine on the Dose Response Curve of Acetylcholine on Isolated Chicken Ileum, Chicken Duodenum and its Comparison With Rat Ileum and Rat Duodenum

Atropine (3.45, 11.40, and 34.55 -nM) shifted the concentration response curve of acetylcholine toward right with a change in EC<sub>50</sub> value [Table 1]. Antagonism produced by atropine was found to be competitive in nature as shown in Schild plots [Figures 2–5]. pA<sub>2</sub> values of atropine had shown significant difference as shown in Table 1. These values

![Figure 1: Effect of acetylcholine on contraction shown by chicken ileum, chicken duodenum, rat ileum, and rat duodenum. Results are expressed as percentage increase in contraction. Values are expressed as mean ± SEM for acetylcholine concentration in tissue preparations, (n=3)](image-url)
The values in the parenthesis indicate EC$_{50}$ range at 95% confidence interval. The values of pA$_2$ are expressed as mean ± SEM (n = 3)

Three- and Four-Point Bioassay of Acetylcholine Using Chicken Duodenum and Chicken Ileum Respectively

The potency of unknown sample of acetylcholine by three-point bioassay using chicken duodenum was found to be 16.26 ± 0.32 μg/ml and the potency of unknown sample of acetylcholine by four-point bioassay using chicken ileum was found to be 39.99 ± 0.13 μg/ml.

DISCUSSION

The present study was aimed to develop ex vivo model for pharmacological experimentation, which will mimic the actual laboratory condition without sacrificing the experimental animals. To find the suitability of chicken ileum and duodenum, experiment was designed to construct concentration response curve of acetylcholine alone and in the presence of different concentration of atropine. The same procedure was followed to see the effect of atropine on the concentration response curve of acetylcholine using rat ileum, duodenum, and also to find out the EC$_{50}$ and pA$_2$ value. In case of chicken ileum, it was found that acetylcholine given alone in the tissue bath had shown almost equal EC$_{50}$ value to that of rat ileum. Similarly, in case of chicken duodenum, the EC$_{50}$ value of acetylcholine alone was slightly greater than the rat duodenum. Atropine had antagonized the effect of acetylcholine on isolated muscle preparation in a similar manner. The pA$_2$ values for atropine in chicken ileum were higher than the rat ileum.
Figure 3: (a) Effect of atropine on contractions induced by acetylcholine in chicken duodenum. Results are expressed as percentage decrease in contractions induced by acetylcholine. Values are mean ± SEM of 3 observations for acetylcholine concentrations of atropine. (b) Schild plot for atropine against acetylcholine with slope 1.174 ± 0.09

Figure 4: (a) Effect of atropine on contractions induced by acetylcholine in rat ileum. Results are expressed as percentage decrease in contractions induced by acetylcholine. Values are mean ± S.E.M of 3 observations for acetylcholine concentrations of atropine. (b) Schild plot for atropine against acetylcholine with slope 1.50 ± 0.63

Figure 5: (a) Effect of atropine on contractions induced by acetylcholine in rat duodenum. Results are expressed as percentage decrease in contractions induced by acetylcholine. Values are mean ± S.E.M of 3 observations for acetylcholine concentrations of atropine. (b) Schild plot for atropine against acetylcholine with slope 1.25 ± 0.38
preparation. Similarly, in case of chicken duodenum, higher pA2 value was found as compared to rat duodenum.

As the EC50 of acetylcholine in chicken ileum is almost equal to rat ileum, based on the above facts, we can predict that the sensitivity of chicken and rat ileum is almost equal. However, the pattern of response to acetylcholine and nature of antagonism with the atropine were similar to that of rat ileum preparation. Similar results were obtained with the chicken and rat duodenum. Hence, chicken ileum and duodenum preparation can be used as a substitute for rat and guinea pig ileum preparation to perform pharmacological experiments.

In present study, we used chicken ileum and chicken duodenum to perform three- and four- point bioassay. Both the preparation had given reasonably good response for about 6- to 8- hours. The results revealed that the chicken ileum and duodenum is suitable tool for performing various types of bioassays. Ileum, being relatively more resistant to trauma is easier to set up and has larger contractions; therefore, it is most commonly employed smooth muscle for study of drugs on isolated muscle preparation. Ileum consists of a number of receptors including muscarinic, histaminic, GABAergic, serotonergic, and adrenoreceptors. The advantages of using cock ileum preparation are that it is economical (even available free of cost), very easy to mount in organ bath, gives reasonably good response for about 6- to 8- hours and importantly, without sacrificing the experimental animals.

CONCLUSION

These results suggest that isolated chicken ileum and duodenum preparation can be employed for routine experiments of Pharmacology subject. The preparation is also suitable for carrying out various experiments on isolated ileum and duodenum, like 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.

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